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SIMPLE COLORIMETRIC MICRO-METHOD FOR DETERMINING THE OXYGEN CONTENT OF WATERY SOLUTIONS

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(With One Text-Figure)

CREAS (1935) describes a method for the approximate colorimetric determination issolved oxygen with 2-4-diaminophenol dihydrochloride, using a concentrated tion of this substance and a mixture of cobalt chloride and potassium dichro- e solutions for colour comparison. The colour formed is described as a com- ation of red and yellow, which varies with the concentration of oxygen, but is directly proportional to the oxygen content. It is intended for field work and for accurate determinations.

In this paper we describe the use of 2-4-diaminophenol dihydrochloride for d, accurate micro-determinations of dissolved O_2 in water and salt solutions.

PRINCIPLE

Diaminophenol, in alkaline solution is oxidized by dissolved O_2 to a substance ng a pure blue colour, which is sufficiently stable; the intensity of colour is ctly proportional to the amount of O_2 present in the solution.

REAGENTS

(1) 200 mg. 2-4-diaminophenol dihydrochloride (Eastman P 614) is dissolved ml. 0·1 *N* HCl; the weak red colour which may be present is reduced by a few stals of sodium hydrosulphite, of which as little as possible is used. Then 25 ml. ure glycerol are added, and the colourless solution is stored in an automatic ette or similar vessel, so that 0·01 ml. samples can be taken. After 2–3 weeks, glycerol solution acquires a yellowish brown colour, and although in the presence xygen the blue colour still develops quite well, the yellow colour interferes htly with a standard calibration curve, which has been made without its presence; storage vessel is therefore emptied, rinsed with alcohol and filled with a freshly pared solution.

(2) 250 mg. NaCN are dissolved in 2 ml. distilled water and 25 ml. glycerol are led. This solution can be kept for long periods and is stored in another, similar ette. This solution is used for stabilization of the blue colour which develops oxidation of the diaminophenol.

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* The publication of this paper has been delayed owing to war conditions; authors' corrections e proof have not been available.

(3) 10% Na_2CO_3 solution. This solution is diluted 10 times when used, 1% solution of carbonate being a suitable medium for the ultimate oxidation o diaminophenol.

(4) Evacuated distilled water or salt solution, stored over mercury in a suitabl vessel, so that samples may be taken without contact with O_2.

(5) A container with commercial carbon dioxide gas, with rubber tube an glass capillary.

(6) A small mercury container which will deliver drops of mercury.

The colorimetric estimation is made in a small cylindrical glass-stoppered tub (content 0·2 ml.), which will fit in a vertical hole drilled into a copper disk, inserte in front of the lamp of a photoelectric colorimeter. This disk has two slits, on corresponding to the hole containing the sample tube, the other being a control slit Both slits can be closed alternatively by a hinged shutter. The photoelectric colorimeter has already been described (Brinkman & Wildschut, 1938) and consists of stabilized neon lamp with adjustable resistance, suitable simple galvanometer (e.g the "spot" Cambridge galvanometer) and a S.A.F. or other type of photocell Standardization of illumination is effected by the adjustable resistance, exposing the photocell through the control slit, and then the actual determination is made by reversing the shutter. The solution the O_2 content of which is to be measured is collected in a well-greased 1 ml. syringe fitted with a suitable needle. The syringe contains a small metal ball for mixing purposes and is filled air-free with sufficient of the 10% Na_2CO_3 solution to ensure that after taking the sample the final concentration of the carbonate will be 1%.

The filling of the syringe must of course be done by very gentle suction so that the formation of gas bubbles is entirely excluded. For a determination the small glass tube is provided with (*a*) a small drop of mercury, (*b*) 0·01 ml. of diaminophenol solution, (*c*) 0·01 ml. of cyanide solution. The needle of the syringe containing the sample is immersed into the glass tube just under the surface of the reagents; the tube is quickly filled up and stoppered. The surface of the solution, which has been in contact with the gas in the tube, is discarded by the immersion of the conical glass stopper.

If necessary the glass tube can be previously filled with CO_2 by means of a fine capillary so that contact with oxygen is prevented. When filled and stoppered the contents are well mixed; a pure blue colour develops quickly and the tube is placed in the colorimeter. The readings are constant after a few minutes, and will remain so for at least 20 min.

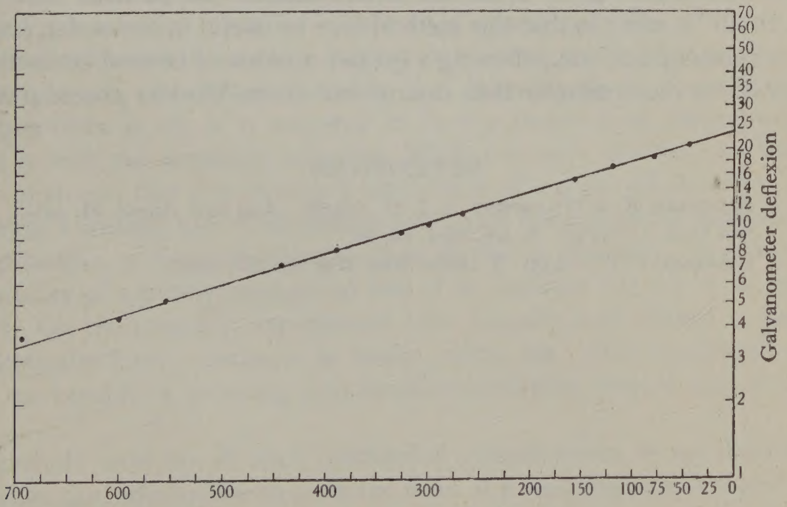
CALIBRATION

This was done (*a*) by making mixtures of air-saturated distilled water and of evacuated water and (*b*) by comparing the colorimetric values to those obtained by van Dam's titrimetric micro-Winkler method (van Dam, 1935). The latter procedure was used for O_2 tensions above atmospheric pressure. The evacuated water was stored over mercury and handled under CO_2 gas; its O_2 content was determined the colorimetric method, assuming a linear relationship between log galvano- er deflexion and O_2 content.

The table below shows the figures obtained for a 0·2 ml. colorimetric tube, nination through the control slit giving constantly 9·0 div., and indicating div. if the tube was filled with pure water.

mm.	O_2 tension	Galvanometer deflexion div.
0		23·8
6	Evacuated water	23·2
44	3 parts evacuated water + 1 part air-saturated water ($t=25·0°$, bar. = 750 mm.)	20·8
83	1 part evacuated water + 1 part air-saturated water ($t=25·0°$, bar. = 750 mm.)	18·4
119	1 part evacuated water + 3parts air-saturated water ($t=25·0°$, bar. = 750 mm.)	16·5
157	Air-saturated water at $t=25·0°$, bar. = 750 mm.	15·2
266	Estimated by v. Dam's micro-Winkler method	11·2
300	,, ,,	10·2
326	,, ,,	9·6
375	,, ,,	8·4
390	,, ,,	8·1
443	,, ,,	7·1
555	,, ,,	5·1
600	,, ,,	4·4
694	,, ,,	3·7

The figures of this table, plotted semilogarithmically in Fig. 1, illustrate the ight-line relation between log galvanometer deflexion and O_2 tension; a



Oxygen tension in mm. Hg (25·0°)

Fig. 1.

ndard curve for any given tube and photoelectric meter may be obtained from dings of pure water and air-saturated water at known temperature and barometric pressure. For determination of oxygen pressures higher than atmospheric pressure and for estimations in sea water the following modifications are necessary.

(*a*) For O_2-rich water 0·02 ml. of the diaminophenol dihydrochloride solution is required; accordingly, the total amount of water in the 0·2 ml. tube will be 5% less. A corresponding correction for this has been applied in the construction of the calibration curve.

(*b*) A very high O_2 tension will easily cause a loss of O_2 during the filling of the tube; it is recommended to dilute such samples by sucking an equal part of evacuated water into the sampling syringe.

(*c*) With sea water the carbonate solution will cause a heavy precipitate. In a narrow 3 ml. centrifuge tube 0·2 ml. of the 10% carbonate solution is covered by 0·5 ml. of pure paraffin oil. 2 ml. of sea water are injected from a syringe with a suitable needle under the oil, the needle being used for gently mixing the carbonate and sea water. After centrifuging for 10 min. the clear solution can be sucked up into a 1 ml. syringe and injected into the measuring tube.

A calibration curve is made by measuring the absorption for sea water saturated with air at known temperature and pressure and the value for pure water. Experiments of this type have shown that a linear relation holds good in this case also.

(*d*) 0·9% NaCl solution or Ringer solution, and also tap water, can be used like distilled water; the faint cloudiness which will appear if 1% of carbonate is dissolved in tap water causes no measurable disturbance.

The average difference in duplicate determinations, over the whole range of oxygen pressures, does not exceed ±0·1 div.; the corresponding deviation in mm. Hg can be seen from Fig. 1. A double determination can be done in 0·6 ml. of water in about 10 min., so that this method may be useful in series determinations of oxygen consumption, etc., allowing a greater number of control estimations. In our opinion it is much simpler than macro- and micro-Winkler procedures.

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NEURAL INDUCTION IN EPIDERMAL EXPLANTS IN LIQUID MEDIUM

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(With One Plate)

previous communications (Waddington, 1938; Shen, 1939), it has been shown t a water-soluble derivative of a carcinogenic hydrocarbon is a potent neurang agent when implanted with the coagulum of egg albumen into a young trula. The present paper describes a similar study with the following modifiions: (1) pieces of completely isolated presumptive epidermis were used instead the whole gastrulae; and (2) the evocatory substance, Na-1:2:5:6-dibenzanacene-α-β-endo-succinate, was not tested in the form of a solid implant in nixture with the coagulum of ovoalbumen, but as a solute dissolved in a neutral solution in which the epidermal explants were cultivated.

The considerations which prompted the present investigation can be sumrized as follows: In normal amphibian gastrulation, the dorsal ectoderm unders neurulation only when and where it becomes underlain by the invaginating soderm. Conversely, where such a mesodermal underlining is altogether wanting, is the case of complete exogastrulation, no neurulation takes place. Similarly, iece of presumptive medullary plate of a young gastrula, when completely ated and cultivated in a neutral salt solution, forms nothing but epidermal cells oltfreter, 1938). It appears therefore that in order that a piece of ectoderm uld neurulate at all, it is essential to have a mesodermal substratum which vides it with the necessary stimulus. While extensive studies have led to the eral conviction that this evocatory stimulus is of a chemical character, there is ertheless a striking lack of one simple and direct demonstration to show that for ral induction, a chemical stimulation is *all* that is necessary. In other words, possibility of a purely mechanical role of an inductor has yet to be decisively luded. On the contrary, experiments have actually been alleged to show that h chemically inert substances as kaolin, silica, etc., when implanted as solid sses, are capable of inducing ectodermal neurulation, even if only to a minor ree.

To exclude once for all such mechanical complications as are inevitably inved when the inductor has to be in the form of a solid implant, it would only be essary to try to induce neurulation in an isolated piece of ectoderm by cultiing it in a fluid medium containing some evocatory substance in a completely ble form.

This is of course by no means a new idea. The earlier attempts of Geinitz 21) and Mangold (1924) (see Holtfreter, 1929) of cultivating fragments of the young gastrula in an organic medium outside the body unfortunately did not lead to any result, as the explants failed to survive long enough for typical differentiations. On the other hand, when the explants were cultivated in the coelom or eye cavities of the older larvae, the ectodermal fragments were found to undergo definite neurulation. But since, in this case, the explants could not but come into constant and intimate contact with the host tissue, one can hardly give all the credit to the body fluid itself, and perhaps none at all. More recently, with improved technique, Holtfreter (1934) again failed to obtain neurulation in ectodermal explants cultivated in chick embryo extract, which, however, becomes powerfully inductive after being heated to form a solid or semi-solid coagulum.

The apparent failure thus far to obtain a definite neural induction from isolated epidermal pieces in the absence of a more or less rigid substratum is highly suggestive of an important, if not indispensable, role of a mechanical stimulation in evoking neural differentiation. In view of the far-reaching significance that this question might bear on the theory of chemical induction in embryonic organization, it seems desirable to re-examine the problem by some further investigation making use of a culture medium containing a soluble material which is chemically pure and has been shown to be potent in evocation when administered as a solid implant into a whole embryo.

MATERIAL AND METHOD

Owing to their comparatively large size, the axolotl, *Ambylostoma mexicanum*, gastrulae were preferred for the present investigation. To avoid any possibility of the ectoderm being influenced by the invaginating mesoderm, only eggs showing the first sign of gastrulation were used. A small disk of the presumptive epidermis was dissected out from the egg at a spot exactly opposite the point of gastrulation. By referring to the maps of presumptive areas of the amphibian gastrula (Vogt, 1929; Holtfreter, 1938), special caution was taken against the inclusion in the explants of any trace of the marginal zone material. The inner surface of the isolated piece was also ascertained to be absolutely free from the adherence of loose yolk cells. Stringent asepsis was observed throughout the operative procedures and the subsequent handling. Each explant was cultivated separately in a small sterilized glass vessel containing 1 c.c. of the culture fluid. Two parallel series of cultures were made: one as control in plain Holtfreter solution; the other in the same containing the hydrocarbon at the concentration of 10 μg./c.c. No attempt was made to vary the concentration of the evocatory substance.

RESULTS

Altogether fifty explants were made, with half the number in each series to be referred to as control and experimental series. All explants in both series were cultivated for not less than 10 days before they were fixed and prepared for histological examination. Within this period, any fragments showing conspicuous sign of infection or degeneration were discarded. Only apparently healthy looking ones were counted.

At the end of 10 days of cultivation, it was found that out of the twenty-five ces of explants in each series, sixteen survived in the control, and eighteen in experimental series. Superficially, the surviving pieces all appeared firm and lthy, with the normal amount of pigmentation. They were generally of a more or spherical form; but some were quite irregular in shape, though without the htest suggestion of any normal morphological orientation.

The first important thing to be noted from a histological study of all the lants from both series was that in none of them was there to be found even a e of mesodermal cells. There were only epidermal cells and, where neurulation k place, nerve cells. In the control series, all surviving explants exhibited the e histological picture, namely, an irregular mass of exclusively epidermal cells random orientation (Fig. 1). In a striking contrast to this, however, eleven of the eighteen surviving explants from the experimental series showed definite quite extensive neurulation, mostly in the clearly recognisable form of neural es in cross-section. The remaining seven pieces of the same series were found ave undergone no further differentiation than mere epidermal cells similar in earance to those found in the control series.

For a closer study of the neural structures induced in the eleven positive cases, y were graphically reconstructed. It was then revealed that these structures e, strictly speaking, not neural 'tubes', but rather 'balls' consisting of radially nged neural cells with their elongated nuclei close to the base. The neural lls' were in most cases hollow in the centre. Some had simple typically shaped ens (Fig. 2). Others, particularly those of relatively large sizes, had their tral cavities of varying complexity (Figs. 3, 4). It is interesting to note that he latter case where the lumens were more complicated than a simple oval hole ross-section, they were usually more or less radially symmetrical in character.

Sense organs or even their rudiments were not observed in any of the explants. cept for a difference in the relative size, there is no convincing way of dis- guishing the induced neural structures as of cephalic or caudal character.

DISCUSSIONS

Admittedly, the total number of the epidermal explants investigated in the sent study is by no means large, but the complete absence of any sign of in- ction in the sixteen surviving explants in the control series in a sharp contrast a high proportion of over 60% of its occurrence in the experimental series, doubtedly adds much weight to the conclusion. Moreover, in all the positive es, the process of neural induction is not represented by the mere formation of a scattered neural cells, but by a well developed neural structure formed by tologically distinct neural elements.

For the present investigation, it is of particular importance that all explants uld be cultivated for at least 10 days before they are ready for histological mination. Not only does a longer period of cultivation enable a better formation orientation of the neural structures, where induced, but also allows ample time for the marginal zone materials, if inadvertently included in the explants, to manifest themselves by their differentiating into clearly recognizable structures of mesodermal or entodermal origin. As already mentioned, in none of the explants examined after 10 days' cultivation, whether in plain salt solution or that containing neurulizing substance, were any mesodermal or entodermal tissues to be found. All explants as they were isolated must therefore be quite free from any but presumptive ectoderm, and possibly presumptive medullary plate. Holtfreter (1938) has proved beyond doubt that the presumptive medullary plate at its early stage is in no way more predisposed toward the formation of nervous substance than is the presumptive epidermis. It is clear, therefore, that there can be no question of either self-differentiation or mesodermal induction of the explants in the experimental series that showed extensive neurulation, which must then be due exclusively to the evocatory action of the hydrocarbon derivative present in the cultivating medium in the experimental series. Nor can the evocatory action of the substance be due to its toxicity, as there were practically equal numbers of survivals of the explants in both series. As far as histological examination can reveal, all but two of the total of thirty-four explants studied appeared to be perfectly healthy. It is of interest to note that in the two specimens which did show signs of partial cellular degeneration, there is no trace of neurulation at all. It is therefore quite unlikely that any of the inductions obtained in the present work might be caused by some disintegrating products of the autolysing cells.

Hence, as far as the evocatory potency of this particular hydrocarbon is concerned, the present investigation not only confirms the finding of the previous one, but substantially strengthens its validity by excluding all uncertain factors, both chemical and mechanical, which were conceivably involved in the earlier method of using the coagulum of egg albumen as the carrier substance.

As mentioned in the introductory remarks, the present undertaking aimed, among other things, at putting to a test the suggestion that for neurulation of the ectoderm, a mesodermal substratum is indispensable for some undetermined mechanical reasons. Spemann (1938), in attempting to make a compromise of the two different views held by Holtfreter and Goerttler as regards the self-determination of the presumptive medullary plate, made the suggestion that the failure of the presumptive medullary plate to neurulate in the exogastrula might be caused by some mechanical deficiency due to the lack of a material substratum, rather than by the absence of an adequate chemical stimulation. The present work clearly shows that a piece of completely isolated ectoderm can be made to neurulate by a suitable chemical stimulus alone. That is to say, the initiation of epidermal neurulation can be quite independent of what mechanical stimulus or facilitation, if any, might be provided by a mesodermal substratum or an artificially implanted mass.

On the other hand, it must be borne in mind that even if all the mechanical stimulation of a solid mass in the process of neural induction could be ignored, there exists nevertheless at least one conceivable and perhaps fundamental difference in the mode of action between that by an evocatory substance in a freely diffusible form and that by a similar substance dispersed in, or even actively

reted by, a group of living cells. In the former case, the substance acts on the
ponsive tissue uniformly at all points. In the latter, it is possible to exercise not
y spatial, but, in the case of active secretion of the evocator, also temporal
ulations. It appears that for the production of a morphological pattern in
bryonic development, there can be at least two varieties of regulation: One
poses a patternless inductor acting on a 'fielded' reactive tissue; the other
umes a non-determined reactive system subjected to the influence of a regulative
uctor. In nature, these two ways are obviously not mutually exclusive. Experi-
ntally, the influence of a 'field' can readily be reduced to a minimum in a piece
isolated tissue. Now, to have such a patternless ectoderm acted upon by an
ally patternless inductor, such as a solution of some evocatory substance, any
ral structure thus induced would then be expected to be quite patternless. In
extreme case, one may expect the whole explant to become a mass of individually
ngated neural cells oriented and grouped in such a way as determined purely by
mechanical forces to which the cells are subjected. That such an ideal case
not been found in practice may be due to many conceivable factors, e.g. the
ying competency of the reacting cells, which might then result in the dominance
those 'resolved' cells over the others, the latter being prevented from further
erentiation. However, such speculations had better be reserved, awaiting
ther investigations. In the present experiment, those neural inductions obtained
wing more or less radially symmetrical structures can be regarded as an approxi-
tion to a patternless induction, or, 'evocation'. The concept of an embryonic
uction as consisting of evocation and individuation (Needham *et al.* 1934)
plies chemically that in addition to an evocatory substance, there might be other
stances modulatory in function so as to give rise to regional differentiations of
otherwise unsegmented structure (Waddington, 1940). It has long been known
t biologically there are such tissues as head-organizers and tail-organizers, the
mical nature of which, however, remains to be explored. Some progress in this
ection has been made by Chuang recently (1938). It should be possible, by
ther simplification of experimental conditions, to put to a crucial test the hypo-
sis of not only chemical evocation, but also chemical modulation.

SUMMARY

1. Epidermal explants of axolotl gastrulae were cultivated for not less than
days in plain salt solution and that containing a water-soluble derivative of a
cinogenetic hydrocarbon, Na-1:2:5:6-dibenzanthracene-α-β-endo-succinate.

2. None of the explants in the control series showed neurulation, while over
% of those in the experimental series showed definite and extensive neural
ferentiation.

3. The induced neural structures were in the form of more or less radially
nmetrical neural balls, with no regional differentiations.

4. By avoiding the use of a solid implant and thus eliminating any possible
chanical role of an inductor, it proves clearly that, as far as evocation is concerned,
chemical action alone of the evocator constitutes an adequate stimulation.

This study was made in the spring of 1939. Its publication has so far been withheld with the hope of including some more observations by extending the study to the various other evocatory substances in the subsequent laying seasons when suitable embryonic material should again be available. Unfortunately, the scheme has not been realized owing to various difficulties. It is therefore decided to put forth now these preliminary observations.

The author most gladly expresses his thanks to Dr C. H. Waddington for his advice and interest in the work.

Note added in proof.

While this paper was in press, an important communication from Barth has appeared (Barth, 1941). Dr Shen saw it only just before his departure for China, and asked me to add a short note, pointing out the relation between Barth's results and his own. Barth's main contribution is to have provided evidence that ectoderm isolated from the amphibian gastrula and cultivated in salt solution may develop into neural tissue even in total absence of any tissues from the organization centre. Such a 'labile determination' of gastrula ectoderm has not previously been satisfactorily demonstrated in the amphibia, although evidence for it has been produced in the chick (Rudnick, 1938; Abercrombie, cited Waddington, 1940). It is important to note that Barth found that the development of neural tissue occurred only if special precautions were taken to preserve the polarity of the isolated tissues, and that its formation was favoured in larger explants; in the latter respect his results are reminiscent of those of Lopashov (1935). His results, however, confirm the earlier conclusions of Holtfreter and others that smallish explants cultivated in the normal way, with no attempts to preserve polarity, do not form neural tissue. The explants made by Shen were of this standard type. His control explants did not develop neural tissue, and there is no reason, on Barth's evidence, why they should have been expected to do so. The formation of such tissue in the treated series therefore remains valid evidence of activity in the substance administered to them.

It would be out of place to attempt here a general discussion of Barth's results, which are obviously, if they can be substantiated, of great importance for the theory of the nature of competence and of the evocator reaction.

C. H. WADDINGTON.

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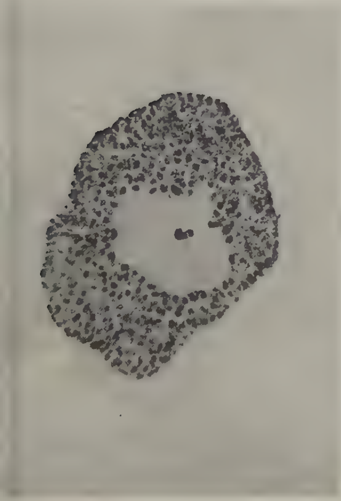

Fig. 1

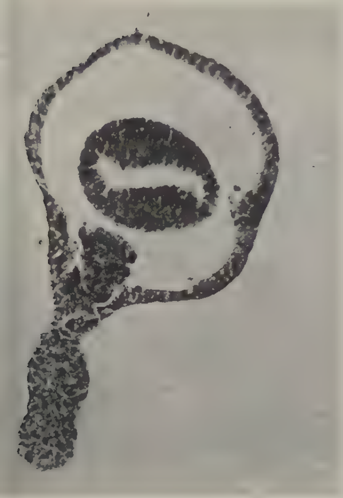

Fig. 2

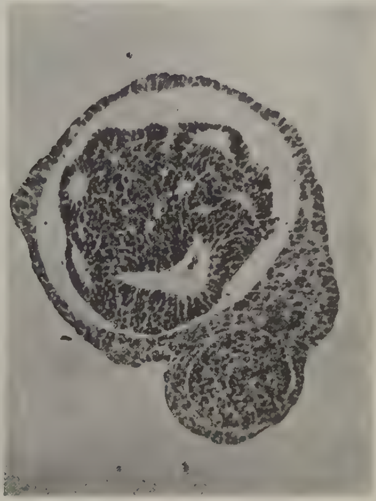

Fig. 3

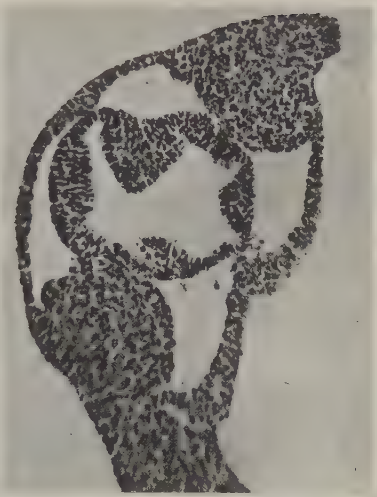

Fig. 4

Fig. 1. Epidermal explant cultivated in plain Holtfreter solution. The explant consists exclusively of epidermal cells.

Figs. 2–4. Neural induction in epidermal explants cultivated in Holtfreter solution containing Na-1:2:5:6-dibenzanthracene-α-β-endo-succinate (10 μg./c.c.). Note the varying complexity of the induced neural structure.

SHEN—NEURAL INDUCTION IN EPIDERMAL EXPLANTS
IN LIQUID MEDIUM (pp. 5–10)

THE LOCALIZATION OF PHOSPHATASE IN CELLS IN TISSUE CULTURES

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(*Received* 1 *September* 1941)

(With One Plate)

a recent paper, Gomori (1941) has described a histochemical method for the alization of phosphatase in animal tissues. The method depends on the fact that enzyme is not destroyed by moderate treatment with 80 % alcohol, in which tissues are fixed. After fixation the tissues are sectioned; the sections are in-ated with sodium glycerophosphate and calcium chloride in buffered solution calcium phosphate is deposited at the site of phosphatase activity. This can rendered visible by treatment with cobalt nitrate solution followed by ammonium phide, when the site of the phosphatase is marked by a black precipitate. By the of such methods he has described, among other things, the occurrence of osphatase in capillary endothelial cells in different animals, not with absolute ularity, but in a large number of cases. In those instances in which a positive ction was not obtained it was shown to be an idiosyncrasy of the particular tissue her than a failure of the method. It seemed possible therefore that an attempt to arate endothelial cells from other mesenchymal cells growing in vitro might be isted by the use of this method.

Hanging-drop tissue cultures were made from 8 or 9-day chick embryos and rt tissue was selected for its content of endothelium. The tissues were explanted a fluid medium consisting of the exudate derived from a clot formed from equal ts of cock plasma and embryo extract. In this medium heart tissue grows very l as a single layer of cells on the surface of the coverslip. The outgrowth is doubtedly mixed and shows regions of fibroblast-like growth and others where uctures resembling endothelial membranes are formed. Myoblasts, fibroblasts, dothelial cells, histiocytes and undifferentiated mesenchyme cells are among the nstituents which may take part in the formation of the outgrowth where they are an ideal condition for histological examination.

Such cultures were grown for 24 or 48 hr. and then washed in Ringer solution free them from the protein-containing medium. They were then fixed in 80 % ohol for from 4 to 24 hr., brought down to water and then divided into three groups. e cultures in group 1 were incubated in water for $1\frac{1}{2}$ hr. at 37° C. Those in group ere incubated for the same time in calcium chloride and sodium barbital, while those of the third group were incubated in sodium glycerophosphate, sodium barbital and calcium chloride. The actual solutions used were:

Group 1	Group 2		Group 3	
Distilled water	2 % $CaCl_2$	1 part	2 % $CaCl_2$	1 part
	2 % Barbital	2 parts	2 % barbital	2 parts
	Distilled water	7 parts	2 % sodium glycerophosphate	2 parts
			Distilled water	5 parts

At first sight the results on tissue cultures appeared to be disappointing. Most of the cells in group 3 cultures were browner than those in group 2, but there was no one class of cells which constantly showed any markedly positive reaction. In those cells which had well-developed centrospheres, the centrosphere was noticeably brown in appearance, but there was no indication that any particular cell type contained any quantity of active phosphatase. However, an examination of the cells which were undergoing mitosis at the time of fixation brought out some interesting points.

In the accompanying illustrations are shown different stages of mitosis as seen in cultures incubated with calcium chloride alone (group 2) which show the distribution of existing phosphates, and also as seen in similar cultures incubated with sodium glycerophosphate as well (group 3), indicating the presence of phosphates produced during incubation and therefore probably due to the action of phosphatase. It is quite clear that the chromosomes have become heavily coated and that the cytoplasm of the dividing cells also shows a conspicuous darkening. The cell membrane appears to be separated from the cytoplasm by a clear and colourless area which does not appear in group 2 cultures and seems to be due, in part at least, to an impregnation of the cell surface which does not seem to occur in control cultures. Whether there is actually an ectoplasm and an endoplasm in these cells is not certain; it may well be an artefact brought about by the alcohol fixation and the subsequent treatment, but there can be little doubt that the phosphatase reaction shows up the chromosomes, the inner mass of cytoplasm, the centriole sometimes, and the cell membrane of the dividing cell. In most resting cells the nucleoli show some tendency to be blackened, but otherwise the cells remain uniformly negative, except as already stated for some conspicuous centrospheres.

It is tempting to connect the phosphatase activity here described with the reorganization of the nucleic acid and nucleoprotein content of the cell which takes place at mitosis, but it remains for future work to elucidate the true significance of its occurrence. Living cultures incubated with a drop of 0·2 % sodium glycerophosphate in Ringer solution added to a drop of the usual exudate medium grew well, but on subsequent fixation and treatment with cobalt nitrate and ammonium sulphide they showed no difference from the controls, so that, as might be expected, the accumulation of calcium phosphate which occurs in the fixed cells is an artificial phenomenon, and normally the final product of the phosphatase activity is not calcium phosphate, whatever else it may be.

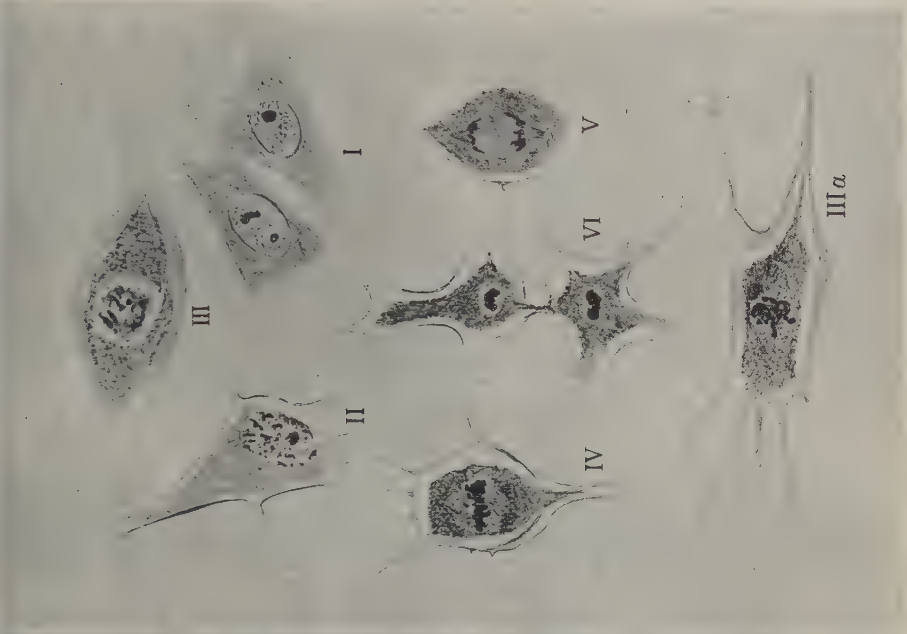

Fig. 2

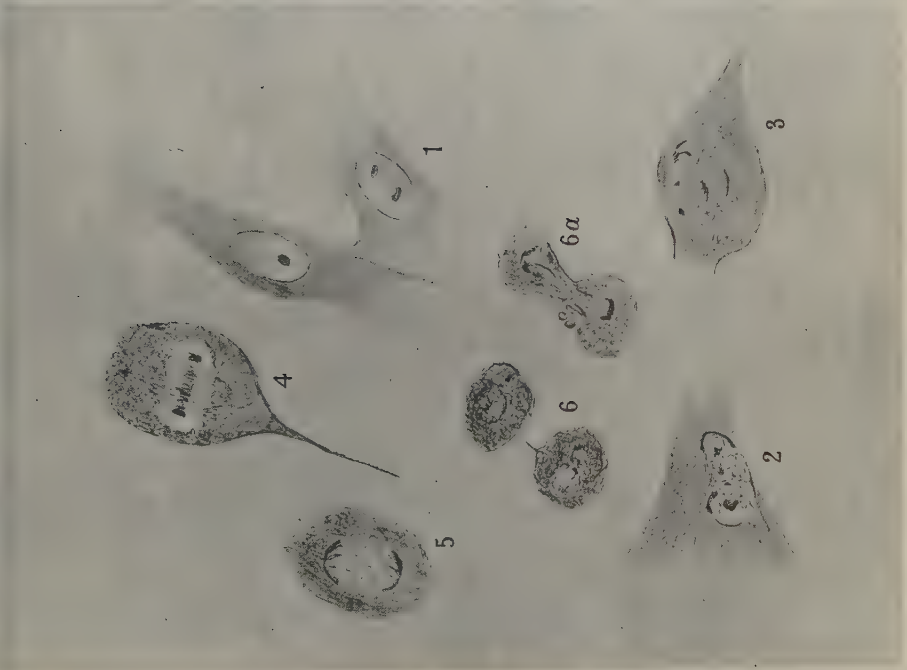

Fig. 1

LLMER—THE LOCALIZATION OF PHOSPHATASE IN CELLS IN TISSUE CULTURES (pp. 11–13)

SUMMARY

The chromosomes of dividing cells give a strongly positive reaction for the sence of phosphatase.

The experiments reported in this paper were carried out with the help of funds eived from the British Empire Cancer Campaign, to whom the author wishes record his gratitude.

REFERENCE

Gomori, G. (1941). *J. cell. comp. Physiol.* **17**, 71.

EXPLANATION OF PLATE 1

Plate I

the left, Figs. 1–6*a* show drawings of cells as seen after incubation with calcium chloride and sequent treatment for the demonstration of phosphates. Controls.

the right, Figs. I–VI show drawings of cells as seen after incubation with calcium chloride and ium glycerophosphate, followed by treatment for the demonstration of phosphates. Positive sphatase reaction.

numbers indicate corresponding stages of cell division. 1 and I, Resting cells. 2 and II, Early phase. 3 and III, III*a*, Late prophase. 4 and IV, Metaphase. 5 and V, Anaphase. 6, 6*a* and VI, ophase.

THE RATE OF REGENERATION OF NERVE

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(With Seven Text-figures)

INTRODUCTION

VERY varied estimates of the rate of nervous regeneration have been given by clinical and experimental investigators, but no one has applied the various possible methods in such a way as to enable comparison of their results. We have therefore made estimates by five different methods in the rabbit. The results showed consistent differences between the rate as estimated in various ways, and consideration of these differences has shown that the techniques do not all measure the same quantity, so that it is not surprising that they appear to reveal different rates of regeneration.

For purposes of analysis we may consider that before recovery takes place after suture of the cut ends of a divided nerve the regenerating fibres must pass through three phases. (1) They undergo retrograde degeneration, branching, and the relatively slow process of outgrowth across the suture scar. (2) The tips of the axons advance down the peripheral stump and make connexion with the end-organ. (3) The newly formed fibres increase from their first tiny diameter and acquire myelin sheaths. Since most of the important sensory and motor functions depend on the conduction of suitably timed volleys of nerve impulses it is only after the processes under the third heading have reached a certain stage that function returns.

The factors which determine the time which elapses between suture and recovery are therefore (1) the scar delay or latent period, including the time taken by the processes under the first of the above headings and that which elapses before the third process begins; (2) the rate of progress down the nerve of the processes of maturation or functional completion of the axons. The rate of progress of the tips of the axons is thus not usually a limiting factor in the time necessary for regeneration, nor is the time taken for the establishment of a connexion between the axon tip and the end-organ. Nothing is known about the duration of this latter period, and throughout this paper it has been assumed that it is shorter than the period necessary for completion of maturation of the regenerated nerve to a level at which function is possible (see p. 41).

It will be seen at once that the concept 'rate of regeneration' is far from simple. have already to distinguish between the apparently simple but clinically less portant process of advance of the axon tips down the nerve, and the more obscure, important, process of advance of capacity to function. If the first alone is sidered as the 'rate of regeneration' then there will appear to be a delay after arrival of fibres at a muscle or sense organ but before the return of reflex ctioning. If the spread along the nerve of the process by which the axons elop to a state at which they can function takes place at the same rate as the wth of the axon tips, then the apparent period of delay after arrival of fibres before functioning will be constant, whatever the distance of the lesion from the -organ. But there is no a priori reason to suppose that either the delay at the per end or the spread of the maturation should take place at the same rates, the experiments detailed below indicate that the delays and rates are in fact erent, the advance of the axon tips being the faster.

A further very serious complication is that the degree of maturation of nerve es which is necessary to ensure functioning may differ, not only for different scles and sensory functions but also, perhaps, with the distance of the lesion m any particular end-organ. A relatively little developed stretch of nerve may fice for conduction over short distances, where the time scatter of impulses not become large. We are faced therefore with the possibility that the entity ose rate of advance down the nerve we wish to measure may vary with the tance of the lesion from the end-organ. If this is so no fixed 'rate of eneration' will appear, the apparent rate being constant only for the recovery a particular function after lesions at given levels.

Consideration of these complications makes it apparent that when measuring rates of progress of the processes of regeneration down the nerve it is necessary define most carefully the function by whose return the advancing front of re-
neration is recorded. And this study of the rate of regeneration as estimated by ious methods has shown in fact that the apparent rate varies according to the ction chosen as an index for recovery.

In any case it is clear that in order to be able to forecast the date at which overy will occur after a nerve lesion in an animal or man it is necessary to know arately the rates at which the various processes proceed. For instance, the delay the scar is probably constant whatever the level of the lesion, whereas the time en by fibre outgrowth and maturation of course increases with the distance to covered. The 'rate of growth' of nerve fibres cannot be determined simply by iding the distance from lesion to end-organ by the time taken for recovery.

The experiments described in this paper were designed to estimate the rates of various stages of the processes of regeneration. The distinction between the e of advance of axon tip and the rate of advance of functional completion was not preciated when the experiments were planned, but became apparent when it s seen that there were conspicuous differences in the rates recorded by the use of ferent methods.

The nerves of the leg of the rabbit have been used for nearly all the observations.

They are very suitable for such experiments because the tibial, peroneal and sural (saphenous minor) divisions of the sciatic can be separated almost to the top of the thigh, allowing any of them to be divided at this level. After interruption of all three divisions rabbits always develop severe sores on the heel and foot, often leading to serious infection. These developments, which have troubled many investigators, can be almost completely avoided if the small sural division is left intact, since this nerve carries sensory fibres from the region of the heel.

All operations were performed aseptically after anaesthesia, usually with intravenous nembutal, followed if necessary by ether. The nerves in the thigh can be very fully exposed by an incision along the line of the femur, followed by retraction of the biceps group of muscles. If necessary the biceps tendon and fascia can be cut away from the knee and the whole muscle reflected. The rabbits used were adults (except for experiments with newborns) and were of various races. As far as possible animals in good health only were taken, but no special attention was applied to uniformity in weight.

I. RATE OF GROWTH OF SENSORY FIBRES

Methods of three types have been used to estimate the rate of growth of sensory (pain) fibres. (1) Since reflex responses can be obtained by stimulating the young fibres in the nerve itself, the animal may be opened under anaesthesia at a given time after nerve suture and the nerve stimulated by pinching or electrical means to discover the farthest point to which fibres have reached (Young & Medawar, 1940). (2) Sutures of a given nerve may be made at various distances from a certain skin area and comparison made between the times which elapse before appearance of pain sensibility in that area. (3) After sensory recovery has begun in an analgesic area, the rate at which that area shrinks gives a measure of the rate of growth of the sensory fibres.

It must not be assumed that these three methods all measure exactly the same quantity. Whereas the first type of experiment measures approximately the growth rate of the fastest growing axon tips, the second and third measure the advance down the nerve of the process of maturation of such numbers of the new fibres as are necessary to give reflex functioning (see p. 37).

Estimates have been made by all the three methods, but the present paper i concerned mainly with those made by the first. The results obtained by L. Guttman and E. Gutmann with the other two methods will be reported in detail later, th main results only being given here for comparison.

A. *Methods of following the advance of reflex excitability down the recovering nerve*

Estimation of the distance reached by new fibres by means of mechanica stimulation of the nerve can give very consistent results if suitable precautions ar observed. An animal on which, say, the tibial nerve was cut and sutured 25 day earlier is anaesthetized, and the nerve exposed from the level of the lesion down rds to the heel. With a fine forceps it is then firmly pinched at its most peripheral int. If no fibres are present here there will of course be no response to this mulus, and the pinch is then repeated a few millimetres higher, and so on at ervals up the nerve. When the point at which fibres are present is reached an ambiguous reflex response is obtained, usually in several muscle groups. Of urse if the anaesthesia is very deep no visible reflexes are seen, and it is therefore portant in all cases to make a control by exposing some nerve known to be intact pinching this, before investigating the nerve which has been sutured.

It might be expected that responses of gradually increasing intensity would be ained at the tip of the regenerated region. In practice such a zone, though ectable, is short, the threshold between the responsive and unresponsive parts the nerve being very sharp. Measurement of the distance which fibres have wn from the lesion can therefore be made to the nearest one or two millimetres, he pinches are made sufficiently close together on the nerve. As an example of accuracy of the method: in rabbit 191 the peroneal nerve was cut and sutured 22 May 1940 and on 6 June 1940 the nerve was explored under nembutal esthesia. Pinching upwards from the heel gave no response at 41·0 or 39·0 mm. m the suture point, but at 38·0 mm. there was a slight but definite head movement. Two still higher pinches for confirmation gave movements slightly greater n the first. Three separate checks of the distance, measured with dividers, gave o, 37·5 and 38·5 mm.

Errors sometimes arise from the fact that pinching of arteries or veins may give to reflex movements of the animal. Moreover, the sensory fibres in question ow the vessels in their distribution, so that it may happen that the nervi vasorum a nerve remain intact even after the fibres of the nerve itself have all been severed. flexes obtained in this way from stimulation of severed nerves are especially rked where large arteries enter the nerve, for instance in the sciatic above the pliteal space or in the anterior or posterior tibial nerves above the heel. It is metimes very striking to see an animal give reflex responses in this way when a ch is given to a nerve known to be severed from the central nervous system, and eems possible that such reflexes have been responsible for some of the reports autogenous regeneration in isolated peripheral stumps.

Errors from this source can be excluded by making it a routine that all collateral nexions of the nerve be severed before the pinching experiment is begun. In t the nerve should be quite free from the lesion downwards, and cut peri-erally.

Histological examination, by Bodian's method, of the most distal regions of the enerating nerves from which responses were obtained, showed good agreement h the results of pinching experiments. It is not easy to be certain histologically the farthest point to which regenerating fibres have reached. The youngest fibres very fine, and growth cones are seen only rarely. However, in a piece of renerating nerve 10 mm. long with the lowest excitable point half-way along it, re were usually found to be numerous fibres at the upper end, none at the lower. metimes even more exact correspondences were obtained, the first fibres being visible at, or just above, the 'first positive pinch'. This histological examinatio certainly shows that the reflex response to pinching the nerve gives an estimate o the farthest point to which a few of the fastest growing fibres have reached. Th fibres which give the response are unmedullated and can hardly have been lai down for more than a few hours. Such very young fibres appear to be particularl easy to stimulate mechanically; witness the great sensitivity of some human neuromas when exposed under local anaesthesia.

On the other hand, such small fibres are rather difficult to stimulate electrically It is not easy to discover exactly the farthest point to which fibres have reached b stimulation with faradic shocks or condenser discharges. Even using fine silve wire electrodes and discharges of relatively large condensers, responses coul usually only be obtained from points higher in the nerve than those shown by th pinching experiments.

B. *Rate of advance of the axon tips*

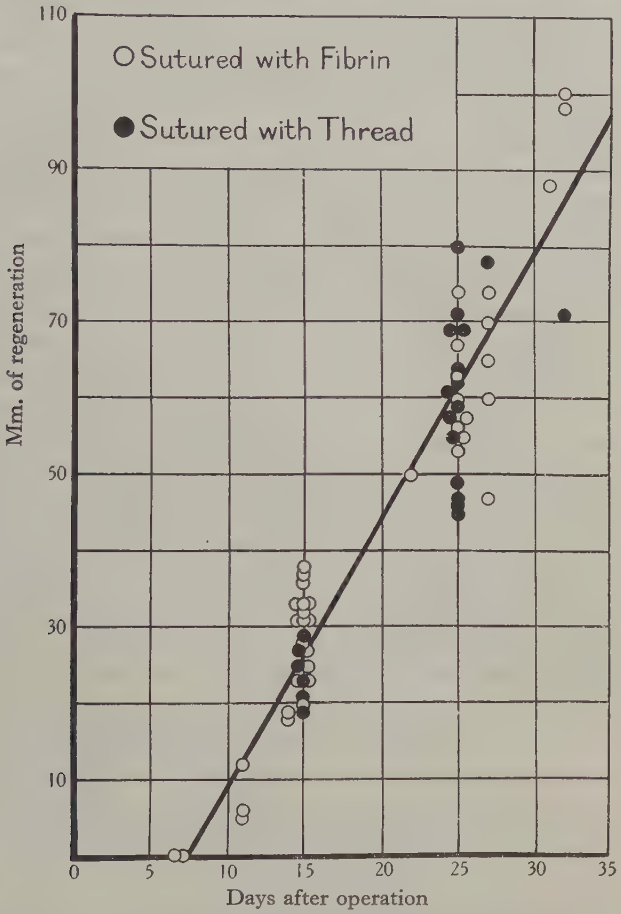
With these precautions experiments by different observers were found to giv reasonably consistent estimates of the distances reached by new fibres after a give number of days. There is of course a considerable scatter; for instance, among th rabbits examined 15 days after plasma suture of the tibial or peroneal nerves distances of outgrowth ranging from 23 to 38 mm. were recorded, with a mean o 30·1 and standard deviation of 5·3. This scatter is greater than would be expecte from phenotypic and genotypic differences alone, but perhaps not more than migh be expected from differences in the making of the suture. There is no reason t suppose that a great part of the scatter is due to observational error.

Fig. 1 shows that beyond all question the distance from the suture point a which responses can be obtained increases with the time between the initia operation and final experiment. This gives the clearest demonstration, on the line of that given by Howell & Huber (1892), that regeneration consists of a proces spreading down the nerve. It appears that after an initial delay in the scar th fibres advance down the nerve at a constant rate. In fact the points of Fig. 1 ma reasonably be fitted by a straight line. However, since, in order to facilitate statistical comparisons, nearly all of the observations were made after either 15 o 25 days, it is not possible, from these data, to test the hypothesis that the rate o growth is constant; in all the following calculations it has been assumed to be so.

C. *The delay in the scar*

The data indicate clearly that during the first few days after suturing no fibre appear in the peripheral stump. Presumably during this period they are undergoing retrograde degeneration for a few millimetres from the injured surface splitting up into branches and advancing across the scar between the stumps Cajal (1928) estimated that the first part of this period, before fibres reach the uppe margin of the scar, was about 2–3 days, and that fibres then grow at a rate o 0·25 mm./day across the scar. He therefore found that axons only appeared in th peripheral stump '7 or more days' after suture in rabbits.

In the present experiments the length of this period has not been carefully ermined by independent experiments, though the points marked on the base- of Fig. 1 show that it could be so determined. If the assumption that the rate rowth in the peripheral stump is constant be correct then the latent period can ily be calculated as the point at which the regression line of distance travelled he new fibres on time cuts the base-line. The data of Fig. 1 thus give as an



1. Plot of the distances reached by new fibres as revealed by pinching the exposed nerve at us times after suture by stitching or with concentrated fowl plasma. The line is the calculated ssion line for all the points considered together.

mate of this delay 7·27 days after plasma suture. Presumably the period will considerably with the closeness of apposition of the stumps when the suture ade, and a large part of the variation in the distances of outgrowth recorded t be due to this cause. Indeed, it is possible to some extent to forecast from rvation of the closeness of apposition at operation, whether the distance reached be large or small. For instance, in rabbit 191 it was recorded at operation that reas the apposition on the right side was 'excellent', that on the left was 'rather less successful'. After 15 days it proved that fibres had reached to 38 mm. on th right but only 33 mm. on the left. Histological examinations confirmed that th union was closer on the right.

Nevertheless, it is probable that a great part of this period of delay is occupie by retrograde degeneration and other preliminary processes. For in experiments i which the nerve was heavily crushed at the original operation (p. 22), rather tha being cut and sutured, the figure for the delay in the scar is 5·23 days, that is to sa only slightly shorter than after suture, although the scar separating the centr axons from the intact peripheral stump is certainly shorter and simpler after a crus than after a cut and suture.

This close correspondence between the latent period after crushing and afte complete division suggests that a definite time is needed for the processes pre paratory to axon regeneration, irrespective of the rate at which this regeneratio takes place. That the bulk of the scattering in the estimates for distances is due t differences in regeneration rates between different animals rather than to difference in latent period can be shown in another way. If the differences were due chiefly t differences in the latent periods, then the scatter (expressed arithmetically as th variance) should be no greater after 25 days than after 15 days, always assumin that the rate of regeneration is constant in each individual. On the other han differences between rates of regeneration would produce a scatter which becam more marked at a greater distance from the point of the lesion, and which ideall would be proportional to this distance. By analysis of the variance at 15 an 25 days it is in fact found that the variance is roughly proportional to the mea distance which the fibres have travelled, so that dividing the variance of the dis tances by the mean distance gives a 'specific factor of variance' which is approx mately the same after 25 days as it is after 15 days. This leads us to suppose that it i individual differences in the rate of regeneration rather than in the latent perio that is responsible for the scatter of the estimates.

These figures for delay would agree with Cajal's estimate of 2–3 days as th period before fibres leave the central stump and enter the scar. It must be reme bered that these estimates give the delay before the tips of the fibres begin advance down the peripheral stump. There are strong indications that there is further delay before the process of medullation also begins to advance (see p. 4

D. *Rate of growth after suturing*

Having crossed the scar the fibres grow down the peripheral stump at a great increased rate. The revised estimates of rate of growth of sensory fibres no obtained, and including new data, differ a little, though not significantly, from th provisional ones recorded by Young & Medawar (1940).

The regression coefficients obtained from the data of Fig. 1 show that the rat in twenty-two experiments in which either the tibial or the peroneal was cut a sutured with white or black silk was 3·42 mm./day, whereas in the forty experimen in which the suture was made with concentrated fowl plasma it was 3·53 mm./da le 1. *The results of estimation of the rate of nervous regeneration by various methods in the rabbit. Where alternative estimates, obtained from the pooled data of a number of animals and the paired data from opposite sides of the same animal, are given, the figure which is to be preferred is italicized*

Quantity measured	No. of readings	Rate of progress ± standard error mm./day	Latent period
Rate of advance of tips of pain fibres in nerve			
11 After nerve suture	62	3·45 ± 0·16	7·27
111 Stitch sutures	22	3·42 ± 0·41	7·68
112 Fibrin sutures	40	3·53 ± 0·17	7·27
1121 in tibial	18	3·44 ± 0·28	7·29
1122 in peroneal	12	3·82 ± 0·25	7·62
1123 peroneal into tibial	10	3·37 ± 0·40	6·58
2 After crushing nerve			
121 Adults	34	4·36 ± 0·24	5·23
1211 High crushes	20	4·48 ± 0·28	5·19
1212 Low crushes	14	4·11 ± 0·41	5·08
122 Babies	11	4·27 ± 0·36	2·35
Rate of advance of functional completion			
1 Sensory function			
211 After crushing peroneal nerve at various levels	13	3·04 ± 0·35	9·76
Paired data	*4 × 2*	*3·4*	*19·0*
212 From shrinkage of anaesthetic area			
2121 After crushing	6	2·05 ± 0·14	—
2122 After suture	6	1·57 ± 0·15	
2 Motor function			
221 From recovery after lesions at different levels			
2211 After crushing			
22111 Adults	38	3·05 ± 0·14	20·77
Paired data	*4 × 2*	*3·1*	*21·6*
22112 Babies: paired data	2 × 2	5·4	10·2
2212 After suture			
22121 Adults	19	2·02 ± 0·32	32·35
Paired data	*4 × 2*	*2·6*	*36·5*
22122 Babies: paired data	2 × 2	2·5	16·2
222 From times of recovery of muscles at different levels			
2221 After crushing	6	2·2	—
2222 After suture	1	1·2	—

le 1 shows that neither the rates, nor the periods of delay in the scar, differ ificantly in the two cases. Table 1 also shows that in eighteen of these experiments hich fibrin junctions were made in the tibial nerve neither the rate nor the nt period differ significantly from the twelve junctions made in the peroneal re. Evidence from interruption of all three parts of the sciatic by a single crush 2) also indicates that there are no large differences in the rate of advance of the of the axons in different nerves. Similarly in ten experiments in which a neal central stump was sutured into a tibial distal one the rate is similar, and the nt period only possibly a little shorter. In this type of experiment the apposition veen the cut ends should be particularly satisfactory because any desired length entral stump is available and perfect apposition can be achieved, without any ion. The similarity of the latent periods therefore further supports the view that this time is mainly occupied with processes of reorganization which are independent o the nature of the lesion and scar between the stumps. Since there are no significan differences after the different types of suture, we may pool all the results and sa that after suture the rate of growth is 3·45 ± 0·16 mm./day and the latent perio 7·27 days.

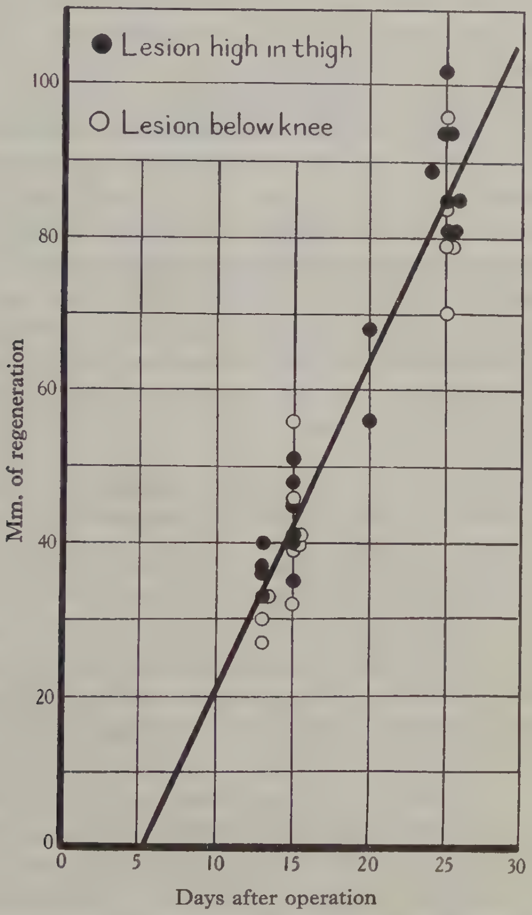
E. *Rate of growth after suture of nerves in the dog*

With the co-operation of Miss E. Bülbring of the Department of Pharmacolog similar experiments were made on three dogs. The sciatic nerves were cut, suture with plasma and explored after 11, 24 and 26 days. In so few experiments it i difficult to gain sufficient experience of the level of anaesthesia required to giv decisive results, but the reflexes were obtained at distances from the suture point which indicate growth rates of the same order as those found in the rabbit. Fo instance, in dog 3 the exploration made after 26 days showed a clear response a 41 mm. from the junction. Histological examination showed that fibres were presen at this point and for some distance beyond it. The delay in the scar cannot hav been less than 8 days, and may have been more, since the junction was not a ver satisfactory one, so the rate of growth of the fibres must have been at least 2·3 mm. day. Similarly dog 4 gave 40 mm. after 24 days, giving a rate of at least 2·5 mm./day

F. *Rate of growth after interruption of a nerve by crushing*

Since even with the most careful technique it is impossible to make unions cut stumps in an absolutely uniform manner in different experiments, it was hope that results showing smaller variability might be obtained by examining the distances reached by new fibres after a nerve had been crushed at one point. Suc crushing, if properly performed, interrupts all the axons, which therefore underg Wallerian degeneration. However, the connective tissues maintain the continuit of the nerve, so that new outgrowth of the severed axons can take place und optimal conditions. In small nerves, such as the peroneal and tibial of the rabbi suitable crushes can be made very effectively by using fine, sharp, smooth-face watchmakers' forceps. Crushing several times at one spot reduces the nerve to transparent thread over a length of 1 or 2 mm. During the following minut opaque matter flows back into the crushed region, so that the site of the crush ca hardly be recognized. Subsequently only a very slight swelling develops at th injured point, and sections suggest that in many cases the new growing fibr remain within their original connective tissue tubes, which were apparently n destroyed by the crushing. Such a lesion must be distinguished from one in whic all the internal structure of the nerve is distorted by the crush so that new fibr have to cross a scar and become criss-crossed much as they would do after sutur Crushing with broad instruments, such as toothed artery forceps, may well produ such disturbances, leaving the ends in a position which may be quite unfavourab for regeneration.

That simple crushes of the first type do completely interrupt all the fibres shown by the fact that the distance from the crush at which reflexes can be elicit eases with time, as after simple suture. Fig. 2 shows the results of thirty-four riments in which the nerve was explored at times varying from 13 to 25 days the initial crush injury. The distances reached are considerably greater than e which would have been found after complete severance and suture of the e, although, as already mentioned, the latent period is hardly different in the



2. Plot of the distances reached by new fibres as recorded by pinching the exposed nerve at is times after interruption of the axons by crushing. The lesions were made either high up or own, but the regression line is that for all the points considered together.

cases. In fact, the greater distances reached after the operation of crushing are to the faster growth of fibres in the peripheral stump (Table 1), the estimate g 4·36 ± 0·24 mm./day.

t is somewhat surprising to find that the rate of growth in the peripheral np varies with the conditions at the point of injury. Presumably the at a suture line presents a region at which the fibres are much attenuated, out endoneural support, and accompanied only by Schwann cells. Being efore thin and ill-nourished they are less well able to grow in the peripheral stump than when they remain throughout in a partially intact nerve, as they d after a crush. It is possible that the crushing interferes less than does cutting wit the blood supply of the stumps, or at least that a good circulation is more rapidl re-established after a crush.

If the rate of outgrowth really depends on conditions between the growing poin and the nerve cell body it is a striking confirmation of the doctrine that regeneratio of nerves consists of an outgrowth from the central stump, in the full sense. Som relatively recent workers (e.g. Spielmeyer, 1929), although admitting that continuity with the nerve cell body is necessary for regeneration, have yet held that th nerve fibre is secreted, as it were, by the activities of the tissues of the periphera stump, and that in this sense the doctrines of Bethe and others of autogenou regeneration are correct.

In any case the fact that the rate of growth in a particular nerve varies with th nature of the interruption is a warning against assuming that such growth rates ca be considered as fixed and unvarying quantities. The high rate of growth afte crushing is itself of interest for the prognosis of recovery in nerves which have bee accidentally crushed at one point in man. It has frequently been observed tha recovery after such lesions is more rapid than after suture, even when the latte is performed under optimal conditions.

G. *Rate of growth after injury at various levels*

It is important to discover whether the rate of outgrowth of new fibres varie with the distance of the lesion from the nerve cell body. Stopford (1920), fro study of the times of recovery after lesions at different levels, has suggested tha the rate of growth of new fibres may be higher when the lesion is closer to the cor

The data analysed above include experiments in which nerves, mostly th peroneals, were crushed at various levels, but so far they have all been considere together. We may now consider separately those crushes made high up and lo down. The highest point which can be satisfactorily reached on the peroneal nerv exposed in the leg is 5–10 mm. below the obturator foramen, and this is the lev described as 'high'. The sensory fibres at this point are about 25 mm. from the cells in the dorsal root ganglia. The 'low' crushes were made in the peroneal nerv reached by a small incision just below the knee. This arrangement gives th maximum difference of level which is experimentally possible with this method the rabbit, namely, up to 120 mm.

Twenty 'high' and fourteen 'low' experiments were made, and as Table shows the rates of growth and latent periods are not significantly different at th two levels. In fourteen cases the nerve was crushed high on one and low on th other side of the same animal. In eleven of these the outgrowth had proceede slightly farther on the side crushed high, but the greatest difference was on 11 mm. This proportion (11–3) would be expected to occur in about 2% of infinite set of trials, and the difference is therefore suggestive of a slightly great rate of outgrowth in the nerves crushed high.

t must be remembered that what is here measured is the rate of growth of the n tips. It may be that the rate of advance of the process of maturation of the e fibres appear to be lower after high lesions than low ones, because the degree mpletion necessary to allow functioning increases with the distance of the lesion n the end-organ.

II. RATE OF ADVANCE OF FUNCTIONAL COMPLETION OF FIBRES

(1) *Recovery in skin after lesions at different levels*

n all the experiments so far described the rate of regeneration has been deter- ed by study of the distance from the point of injury at which reflex responses d be obtained when the nerve itself was exposed and pinched. A completely rate estimate has been obtained by the use of the criterion of the first appearance covery in a given area of skin rendered insensitive by injury. The method has to interrupt the peroneal nerve by crushing at various levels, and then to h for the return of reflex responses to pin prick in the denervated area on the nd foot (Fig. 4). Recovery in such an area of sensory loss is a very complex

le 2. *Times taken for complete sensory recovery after crushing the peroneal nerve at various levels. The distances given are from the lesion to the lowest point of the analgesic area on the foot, and the number of days is that necessary for recovery to reach this point*

Animal	Distance mm.	Days
562	160	67
560	161	70
494	166	68
493	171	68
485	198	67
486	198	90
579	201	70
364	212	94
562	240	90
560	244	91
546	250	96
390	252	85
494	266	101
493	273	101
509	282	95

cess, including overlap as well as regeneration, therefore it is not possible to use datum the moment at which recovery begins at the upper margin. Instead we e used the criterion of recovery at a fixed level, namely, the reappearance of pain ibility in the most distal part of the affected area. The animals were examined kly until recovery had taken place, and the distance from the lesion of this most al part, that is, the dorsum of the second and third toes, was then carefully deter- ed. The details of this investigation will be later described by two of us (E. G. L. G.).

Table 2 and Fig. 3 show the results in the fifteen animals studied. The lesions were made at three levels: 160–170, 195–210 and 240–280 mm. from the most distal point in the area of sensory loss. There is undoubtedly a progression, in that the recoveries took longer after the more proximal lesions, but the data are not so consistent as those obtained by the pinching of exposed nerves. This is only to be expected since more variables may intervene between injury to the nerve and recovery in the skin than when the nerve itself is exposed. Taking all the dat together and calculating the regression coefficient we find a rate of 2·74 ± 0·44 mm. day. That this rate is unduly small is shown by the fact that the correspondin calculated latent period is no more than 3·8 days. The points which seem to fit leas well into the data are those for the middle distance, and if we eliminate the two ver delayed recoveries marked with open circles we obtain a rate of growth c 3·04 ± 0·35 mm./day with a latent period of 9·8 days, which is still a shorter dela than is shown by other and more reliable methods (p. 27).

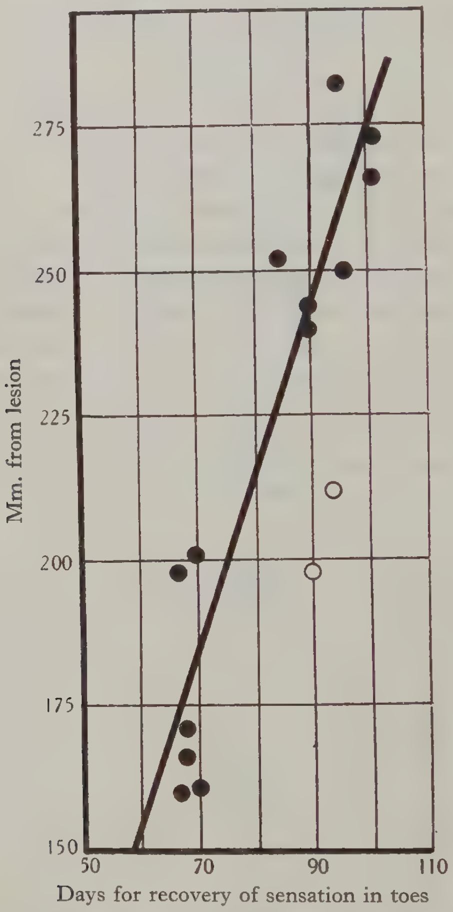


Fig. 3. Plot to show number of days needed for recovery of sensibility at the tips of the toes afte crushing the peroneal nerve at various levels. The two points marked with open circles have no been considered in calculating the regression.

n four of these experiments the nerves were pinched at different levels on the sides of the same animal. As shown by Table 3 comparison of the times of very on the two sides of these animals gives estimates of the rates of recovery h are slightly higher than those obtained from the above regression line, the

e 3. *Comparison of times necessary for complete sensory recovery when peroneal nerve was interrupted high on one side and low on the other side of the same animal. Distances and times calculated as in Table* 2

nimal	Distance mm.	Days	Difference of distance	Difference of days	Rate mm./day	Latent period
560 L	161	70	83	21	3·95	29·2
R	244	91				
562 L	160	67	80	23	3·48	21·0
R	240	90				
93 L	273	101	102	33	3·09	12·7
R	171	68				
94 L	266	101	100	33	3·03	13·2
R	166	68				
				Mean	3·39	19·0

ge of the four experiments giving a rate of 3·39 mm./day. By subtracting the taken for functional completion in the nerve from the total we can then obtain nates of the latent periods from these data (last column of Table 3) and the estimate is 19·0 days. This is the time which elapses before maturation reaches evel of functional completion close to the lesion, and this completion begins to nce down the nerve.

one of these estimates approaches the value of 4·36 given by the method of sing and stimulating the nerve (see p. 23), and it is not necessarily to be cted that they would do so. For the lower figure is an estimate of the advance n the nerve of that process of recovery which makes the nerve able to mediate x functions, and there is no special reason to suppose that the process advances e same rate as the growth of the axon tips.

(2) *Progress of recovery within an insensitive area*

he second method which has been used for estimates of the rate of advance of ional completion of sensory nerve fibres has been to follow the progress of kage of an analgesic area during recovery. A nerve, usually the peroneal, was rupted, and the area of sensory loss mapped by testing the responses of the al to pin prick, marks being made on the skin with silver nitrate (30%) and r photographed or carefully measured and plotted on outline drawings of the Examinations were made, usually at weekly intervals, until there began to be inkage in a zone in which recovery could not be accounted for by overlap from ent nerves. Further examination and measurement then showed the rate at h shrinkage proceeded. There is not a simple steadily advancing front to the tive region; usually the area shrinks from the sides as well as from above downs. Sometimes the downward progression stops while the shrinkage from the sides progresses. But nevertheless, a very definite downward progression can near always be detected. This is especially easy to follow on the dorsum of the foot aft interruption of the peroneal nerve. As the example in Fig. 4 shows, the margin recovery has a ∧ shape and the advance of the apex of the ∧ is very readily followe

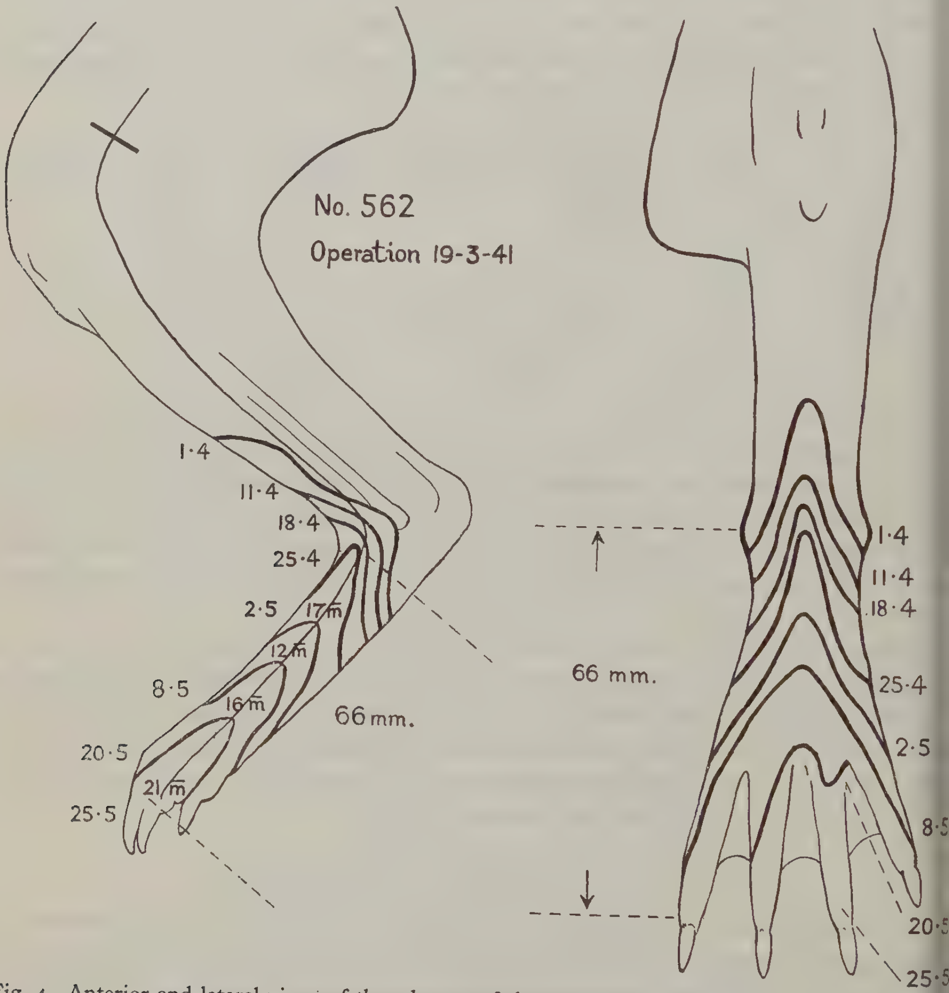


Fig. 4. Anterior and lateral views of the advance of the margin of recovery on the foot of a rabbi after crushing the peroneal nerve at the knee. The course of the nerve and the point of interruptio are shown in the lateral view.

Table 4 summarizes the results in six cases in which recovery was followed afte crushing the peroneal nerve. The average rate of regeneration is 2·05 mm./day but there are large variations between the different animals. Moreover, as Fig. shows, the shrinkage does not always take place steadily throughout the area In fact great care must be taken in deciding which points to choose in making th estimates. In one case the shrinkage in the area supplied by the saphenous nerv proceeded at the high rate of 3·1 mm./day. After suture the advance is still mor le 4. *Rates of growth from progress of recovery on the dorsum of the foot after crushing the peroneal nerve. The distances are those observed after true recovery had begun, and before it had ended* (*see text*)

Animal	Distance on skin mm.	Days	Rate mm./day
560 L	55	22	2·50
494 R	59	31	1·90
562 L	66	30	2·20
579 L	69	36	1·92
546 R	74	49	1·51
509 R	80	35	2·29
		Mean rate 2·05 ± 0·14	

gular and the individual results will be reported later. The average rate given experiments was 1·57±0·15 mm./day.

Before accepting figures obtained in this way as an estimate of the rate of gress of new nerve fibres it must be remembered that the distance between two nts on the surface of the skin does not represent the whole of the distance that ve fibres must travel before the advancing edge of sensitivity moves from the er to the lower point. The distance is greatly increased by the ramifications of cutaneous nerve plexus. It is also possible that regenerative processes proceed e slowly in these ultimate plexuses than in the main nerve trunks.

(3) *Rate of advance of functional completion of motor nerve fibres*

In order to establish the rate of growth of motor nerve fibres it is necessary to pare the times of recovery of motor function after lesions at various distances n the muscle. The function chosen was the spreading of the toes of the rabbit, ovement which can be elicited by holding the animal by the scruff of the neck lowering it suddenly. The spreading is produced by the action of the small oneal muscles II–IV, which are innervated by the peroneal division of the ic. Abduction of the first toe is also produced by the m. abductor digiti primi,[1] ch is innervated by the tibial nerve.

After interruption of the peroneal nerve the absence of impulses tending to ad the toes produces a characteristic narrow appearance of the whole foot, when the animal is suddenly lowered the only spreading is an abduction of the toe. The first signs of recovery, usually abduction of the fourth toe, can thus lily be detected. Daily examination of the animals can be made very quickly. overy after crushing a nerve occurs unambiguously, with an error of as little as ay. After division and suture, however, the earliest signs of recovery remain btful for 2–4 days.

Motor recovery after crushing a nerve.

At an initial aseptic operation the peroneal nerve was crushed on both sides of rabbit, but at varying distances from the muscles. Each distance was measured approximately at the time of operation and then confirmed to the nearest millimetr after recovery had taken place by direct measurement on the exposed nerve. Thirty eight nerves were interrupted in this way, at distances varying from 8 to 110 mm from the muscle, recovery times varying correspondingly from 24 to 58 day Table 5 and Fig. 5 show the results. It will be seen that the period necessary fo recovery after a lesion at any given distance from the muscle is reasonably constant

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[1] The muscle called by Krause (1884), m. extensor digiti primi.

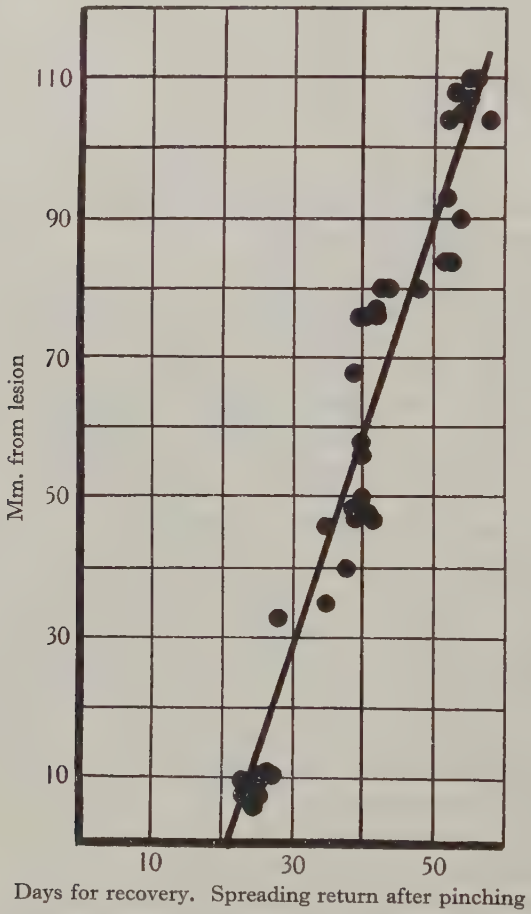

Fig. 5. Plot to show the time necessary for recovery of spreading of the toes after crushing th peroneal nerve at various distances from the muscle.

For instance, for distance 8–10 mm. the times of recoveries vary only from 23 t 26 days. For 40–50 mm. the variation is 35–40 days, and for 100–110 mm. 52–5 days. The regression coefficient of distance from muscle on days necessary fo recovery gives us an estimate of the rate of growth of functionally complete moto fibres, and the figure is 3·05 ± 0·14 mm./day, with a latent period of 20·8 days. I four cases, shown in Table 6, the recovery was followed after the peroneal nerve ha been crushed high on one side of the animal and low on the other. The average rat calculated for these four animals is 3·1 mm./day, with a latent period of 21·6 days.

latent period of 21 days is that necessary for the reaching of functional letion at the point of the lesion, and agrees with the 19·0 days found for sensory s (see p. 27).

e 5. *Times necessary for motor recovery (first appearance of spreading of the toes) after crushing the peroneal nerve*

Animal	Distance mm.	Days	Animal	Distance mm.	Days
493	8	24	522	68	39
494	8	23	536	76	42
567	8	25	546	76	40
542	10	25	588	76	40
566	10	23	636	77	42
579	10	24	635	80	43
562	10	26	623	80	43
560	10	25	390	80	48
579	33	28	468	84	52
578	35	35	468	84	52
584	40	38	562	90	54
486	46	35	560	93	52
422	48	40	509	104	58
422	48	40	561	104	52
421	48	40	561	105	54
421	48	40	507	107	55
485	50	40	508	108	53
364	56	40	493	110	55
363	58	40	494	110	56

Rate 3·05 ± 0·14 mm./day. Latent period 20·8 days.

suggesting that the rates of advance down the nerve are those of a process aturation which moves less fast than the tips of the axons it has been ned that there is no considerable extra delay in the muscle which has been vated for the longer period. This assumption needs to be checked, since it is nlikely that after a long period of denervation and hence of atrophy this delay

e 6. *Time necessary for recovery of spreading of toes after peroneal nerve crushed igh up on one side and low down on the other side of the same animal*

nimal	Distance mm.	Days	Difference of distance	Difference of time	Rate mm./day	Latent period
93 R	8	24	102	31	3·29	21·57
L	110	55				
94 R	8	23	102	33	3·09	20·41
L	110	56				
62 L	10	26	80	28	2·86	22·52
R	90	54				
60 L	10	25	83	27	3·07	21·72
R	93	52				
				Mean	3·1	21·6

l be greater than after denervation for, say, only 25 days. If this proved to e case then the whole of the above calculation would be upset, the length of traversed not being then the only variable. In other words, since we are the return of muscular response as the criterion of arrival of fibres that have started at different distances, we must be sure that this criterion is the same in experiments.

This possible source of error has been controlled by first interrupting the ner high on one side of a rabbit and low on the other, and then at about the time whe it was calculated that motor fibres have grown from the high lesion down to the lev of the low one, interrupting the latter again. The fibres should then start aga level and will reach muscles which have been denervated for equal times. To put another way the first operation on the low operated side serves merely to prepa the muscle on that side, so that it shall be similar, as an index, to its opposit Table 7 shows the results in the three animals treated in this way. It will be see that function returned after approximately the same times of total denervation the two sides, giving rates of growth of the same order as those obtained from th above regression line. The times taken for recovery after the double lesions (di tances 40–46 mm.), were 36–39 days, and are quite comparable to the time required for recovery in the two experiments in which single lesions were made this level (35 and 38 days). The average rate of growth given by these three exper ments, 3·5 mm./day, is slightly higher than that given by the thirty-eight exper ments in which the nerves were crushed once only, and this difference is wh would be expected if the period of delay between arrival of fibres at the musc

Table 7. *Time taken for motor recovery after peroneal nerve crushed high up on o side and on the other crushed twice to ensure the same total period of denervatio on both sides*

Animal	Distance mm.	Days	Difference of distance	Difference of time	Rate mm./day	Latent period
507 L	46	36	61	19	3·21	21·7
R	107	55				
508 L	45	37	63	16	3·94	25·6
R	108	53				
509 L	40	39	64	19	3·37	27·1
R	104	58				
				Mean	3·5	25

and the return of function is greater after longer periods of denervation. While th difference is slight, 3·0 against 3·5, and cannot be tested statistically, it is perha just suggestive of a prolongation of the period of delay with progressive atrophy the muscle. For our present purpose we may draw the conclusion that the rate advance of functionally complete motor fibres after a nerve has been crushe cannot be less than 3 mm./day and is almost certainly rather greater.

There are two possible sources of error in these control experiments themselve which must be mentioned. (1) After the second interruption the outgrowth fibres might be less (or conceivably more) vigorous than after an initial lesion. Bu in other experiments (Holmes & Young) the outgrowth has been found to be at a

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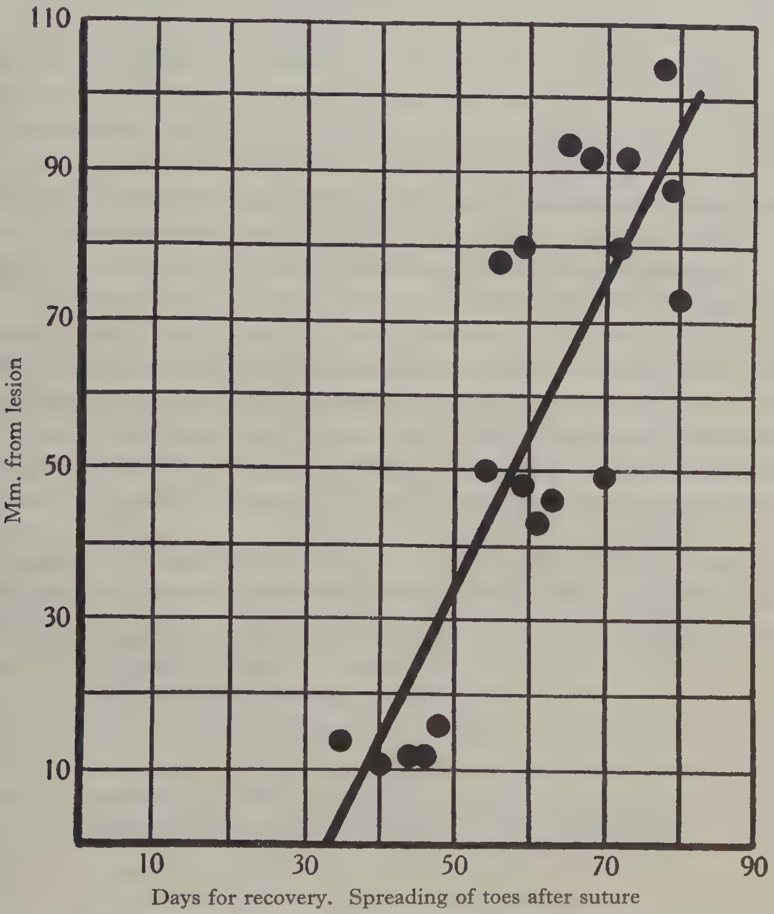
[1] Since the distance between the two lesions was greater than that of the lower one from th muscle it is clear that for a few days before the second operation axon tips must have been present the muscle on the low-operated side, and might possibly have had some effect on the condition the muscle fibres.

s equally vigorous under such conditions. (2) The scar resulting from the first
ration might delay the fibres. Other experiments in which high rates of growth
been observed through much more seriously scarred nerves show that this
nlikely. The effect of any such delay would be to increase the apparent rate of
neration, but it can be dismissed as the basis of the high rate actually observed
the absolute times needed for recovery were not greater after these double
after single lesions (see above).



6. Plot to show the times taken for recovery of the spreading of the toes after severance and suture of the peroneal nerve at various levels.

Rate of growth of motor fibres after suture.

This estimation was made by cutting the peroneal nerve at various distances
n the muscle, suturing with plasma, and looking for recovery of the function
preading the toes. Table 8 and Fig. 6 show the results of the nineteen experi-
ts made. The time between operation and recovery was much longer than
r crushing the nerve. For instance, after injury 40–50 mm. from the muscle

Table 8. *Times necessary for motor recovery (first appearance of spreading of the toes after cutting and suture of the peroneal nerve*

Animal	Distance mm.	Days
569	11	40
584	12	46
563	12	44
564	14	35
572	16	48
486	43	61
574	46	63
574	48	59
485	49	70
573	50	54
521	73	80
590	78	56
589	80	59
604	80	72
563	88	79
569	92	73
573	92	68
564	94	65
572	104	78

Rate 2·0 ± 0·3 mm./day. Latent period 32·3.

the times required for recovery were 38–40 days after crushing, 54–70 days after cutting. Histological examination confirmed the existence of variations at the suture line; for instance in rabbit 485, which recovered very late, there was a separation of nearly 3 mm. between the stumps.

In spite of this variation it has been thought best to treat the data as a single body, and the regression coefficient of time for recovery on distance from the muscle gives a rate of growth of 2·02 mm./day and a total latent period of 32·35 days.

In four animals the nerve was cut and sutured high on one side and low on the other of the same animal. The results of these are recorded in Table 9, and they give a somewhat higher estimate of the rate of growth, namely, 2·6 mm./day and a latent period of 36·5 days, which are probably better estimates than those derived from the regression line. In any case we may conclude that, as with sensory fibres, the rate is lower, and the latent period longer, after cutting and suturing the nerve than after interruption by crushing.

Table 9. *Times necessary for recovery of spreading of toes after peroneal nerve has been cut and sutured high on one side and low on the other of the same animal*

Animal	Distance mm.	Days	Difference of distance	Difference of time	Rate mm./day	Latent period
564 L	14	35	80	30	2·67	29·8
R	94	65				
569 L	11	40	81	33	2·45	35·5
R	92	73				
572 L	16	48	88	30	2·93	42·5
R	104	78				
563 L	12	44	76	35	2·17	38·5
R	88	79				
				Mean	2·6	36·5

(3) *Rate of growth of motor fibres measured by times of recovery of different muscles.*

By recording the times at which separate muscles lying at different distances from a lesion recover, we obtain an estimate of rate of growth partly comparable at obtained by watching the progress of recovery in the skin (p. 27). Of the les innervated by the peroneal nerve the peroneus I (longus), which lies st, produces pronation of the foot, a function not easy to elicit reflexly, and fore not used for our present purpose. Mm. peronei II–IV all produce ding of the toes, and all of them lie higher than m. tibialis anterior and m. sor digitorum, which produce dorsiflexion of the foot and toes respectively, ments easily elicited by pricking the plantar surface of the foot.

ince these movements are produced by several muscles it is somewhat difficult cide what distances to take for estimation. The m. peroneus II being the est muscle producing spreading of the fourth toe, and m. tibialis ant. the est to give dorsiflexion, these alone may be considered. The distance between was carefully measured on two medium-sized rabbits, as follows. From a point on the peroneal nerve to the entry of its branch into m. peroneus II the ge distance was 16 mm., to m. tibialis ant. 23 mm. Since we do not know how of the intramuscular course of the nerve must be run before function returns ay, as an approximation, add to these figures the distances between the point try of the nerve to the muscle and the mid-point of the length of the latter. e additions (6 mm. for m. peroneus II and 10 mm. for m. tibialis ant.) give stances of 22 and 33 mm. from our fixed point to the centres of the muscles, ur estimate is then that fibres must grow 11 mm. farther to reach m. tibialis han m. peroneus II. In six rabbits careful examination was made of the times ich spreading and dorsiflexion returned after crushing the peroneal nerve. ery case the spreading returned first, the times between the appearance of the unctions being 5, 5, 4, 6, 4 and 6 days. Taking the mean of this delay as 5 days, he distance as 11 mm. we obtain an estimate of rate of advance of functional y of 2·2 mm./day. This is at best a very rough approximation; indeed, the distances between the muscles make the rabbit unsuitable for studies of this Nevertheless there is a general agreement with the figure arrived at for rate owth of sensory fibres in the skin (p. 27).

n one case the difference between spreading and dorsiflexion was carefully ined after severance and suture of the peroneal nerve and found to be 9 days. would give a growth rate of 1·2 mm./day, clearly lower than that after crushing erve. But no great reliance can be placed on this single case.

III. RATE OF REGENERATION IN YOUNG ANIMALS

few experiments comparable to the above were made to determine the rates nsory and motor regeneration in rabbits about one month old. Twelve nerves crushed in the middle of the thigh in animals 16–22 days after birth. At vals of 6–13 days after operation they were examined by the method described 16, to discover how far from the lesion reflex responses could be elicited by ing the nerve. The results are shown on Fig. 7. It is impossible to fit a single ht line to these points, because the data from the one animal examined after

13 days show a distance of regeneration relatively much greater than any of the others. It is possible that the data from this animal are anomalous, since it become very difficult to make the experiment as the fibres approach the end of the nerve as they do after 13 days in these small animals. For purpose of discussion the results up to 12 days are considered, and Fig. 7 shows the regression line calculated for the first 12 days only. The latent period is obviously very short, the estimate

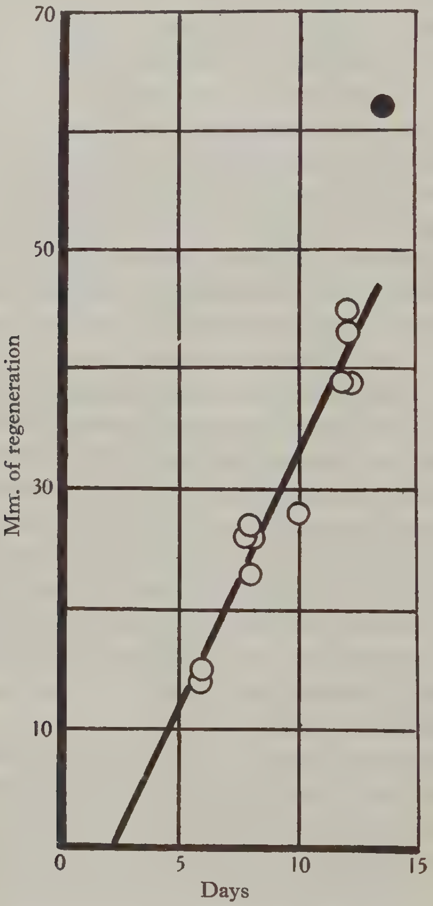


Fig. 7. Plot to show the distances reached by new fibres in animals one month old, as revealed b pinching the exposed nerve at various times after it had been interrupted by crushing.

being 2·4 days, and this is confirmed directly by the nerves examined after 6 and 8 days, in which fibres have grown for considerable distances. On the other hand the rate of advance down the nerve, estimated at 4·3 ± 0·4 mm./day, is of the same order as that found in adults. This is, perhaps, what would be expected, since the processes in the scar, which are essentially those of healing, may well be more rapid in young animals, while the progress of fibres down the nerve trunk, which is as much a movement as a growth process, might be less affected by the age.

Iotor recovery was tested in four animals, of the same age as the above, by upting the nerve high on one side and close to the muscle on the other. The s are shown in Table 10. In the two animals in which the nerves were ed the average estimate is 5·4 mm./day, and when the nerves were cut and ed with plasma 2·5 mm./day. The first of these figures is considerably higher that of 3·1 mm./day estimated for adults, whereas after suturing the nerve the n adults (2·0 mm./day, or more, see p. 34) was little less than in the babies. aken with the results for the rate of growth of sensory fibres in young animals results indicate that the dramatically rapid recoveries made by young animals,

e 10. *Motor recovery (spreading of toes) after interruption of peroneal nerve in rabbits less than one month old*

nimal	Distance mm.	Days	Difference of distance	Difference of time	Rate mm./day	Latent period
			Nerves crushed			
47 L	40	17	35	7	5·00	9·0
R	5	10				
48 R	44	19	40	7	5·71	11·1
L	4	12				
				Mean	5·4	10
			Nerves cut and sutured			
55 R	38	31	31	12	2·58	16·4
L	7	19				
56 R	38	32	31	13	2·38	16·2
L	7	19				
				Mean	2·5	16

children, are not due to very high rates of growth of axon tips in the nerve s, but to great shortening of the delay in the suture scar, and probably eration of functional completion, combined, of course, with the fact that the nces to be covered are small.

DISCUSSION

must be remembered that in no case have the measurements been of the th rate of single fibres; indeed, this would only be possible if the growing tips watched under the microscope as in the experiments of Speidel (1933). In xperiments in which the nerve was pinched to discover how far the new fibres eached, the critical point is that at which a sufficient number, n_1, of sufficiently leted fibres is present. Presumably n_1 is greater than 1, but since histological es have shown that such reflexes are obtained from stretches of nerve in which a few very fine fibres are present (p. 17) it is clear that it represents only a small rtion of the number of pain fibres originally present in the nerve. During eration fibres cross the scar and grow down the stump at varying rates, and es of the distances at which responses to pinching can be obtained give us an ate of the rates for a group close to the fastest.

When a nerve is interrupted by a crush the fibres are all damaged alike and probably regenerate at more uniform rates than after severance and suture of a nerve. Therefore the distance from a lesion at which the number n_1 of fibres necessary to give a 'pinch reflex' is present is probably less after cutting than after crushing. If n_1 was large this statistical fact might account for the greater distances of regeneration recorded after crushing, but since n_1 is known to be small, and since the estimates of growth rate are independent of such factors, we must conclude that the actual rate of advance of axon tips in the peripheral stump is slower after suture than after crushing.

On the other hand, in motor recovery it is probable that the number (n_2) of fibres that must grow down into the muscle before reflex contraction can occur is perhaps half of the original number of motor fibres in the nerve, since many muscle fibres must be innervated before visible reflex movement is produced. Certainly n_2 is greater than n_1 and therefore in the experiment on motor recovery we are investigating not the fibres that cross the scar first and grow fastest, but the rate of some fibres about the middle of the distribution. This will have the effect of making all estimates of motor fibre growth rate appear lower than those for sensory fibres as determined by pinching the nerve. But since we do not know the extent of the scatter of the rates it is impossible to say whether this factor can account for the greater part of the observed difference.

Probably a more important point is that the estimate of sensory fibre growth rate by pinching is based on the point in the nerve to which a small number of very thin and scantily medullated fibres have grown. But the estimate for motor fibres is of the rate, not of the almost unmedullated tips, but of the progress of the process of growth in diameter and of medullation. The fact that studies of recovery, both sensory and motor, give estimates of rates of regeneration close to 3 mm./day (after crushing), compared with the 4·4 mm./day found by pinching the exposed regenerating nerve, suggests that the lower rate is that at which this process of maturation to functional completion proceeds. The control experiments described on p. 32 have shown that the relatively slow rate of growth recorded in studies of motor recovery is not the result of any significant delay in the re-establishment of the function of muscles due to greater atrophy in those which have been denervated for long periods. There remains, however, the possibility mentioned on p. 15 that the degree of maturation of nerve fibres necessary for functioning is less when a lesion is close to a muscle than when it is far away. If such close lesions recover relatively quickly then the estimates of rate of advance of functional completion along the nerve will be too low.

Since both of these two factors which will tend to produce low estimates of growth rate are of unknown magnitude the conclusion that functional completion spreads down the nerve more slowly than does growth of the axon tips can at present be only provisional. The important point however is to recognize that there is such a spread along the nerve, and that what is being measured when sensory or motor recovery is recorded is the rate of spread of this full nervous regeneration not that of the axon tips alone. Our estimates give about 2·0 mm./day after suture il 3·0 mm./day after crushing as the rate of this advance for both sensory and tor functions.

The average figure for the rate of shrinkage of an analgesic area, 2·1 mm./day, er crushing the peroneal nerve, is still lower than any of the above estimates of wth rate. This would seem to indicate either that the nervous pathways between points on the skin are much longer than the measured distance, or that a slower wth process occurs at the periphery. Indeed both may be true.

On this evidence the conclusion is that the actual growth rate of the axon tips s not vary between the different functional fibre types, in all of which it probably ances, in the fastest fibres, at about 3·5 mm./day after suture and 4·5 mm./day er crushing of a nerve. In all cases advance of recovery down the nerve has been ter after crushing than after severance and suture. The data indicate that the ater rate is due not only to less interference with the pathways but to a greater e of advance of fibre tips, and probably of maturation. Though no critical data this point exist in the literature, quick recoveries after crushing have frequently en recorded (e.g. in 19 days after crushing the peroneal in the cat (Langley, 8)) and are fortunately a commonplace of surgical practice.

Whatever may be the explanation for the apparent differences in rates of wth the point of practical importance is that when using different methods in the ne animal such differences have appeared with great regularity. Estimates of eneration made clinically with similar methods are likely to yield similar ferences of apparent rate. A surgeon stimulating exposed nerves under local esthesia may find rates of growth after suture approaching the 3·5 mm./day orded in the above pinching experiments. Studies of the progress of Tinel's n, since they depend on the stimulation of relatively few and little-medullated res, are likely to give high rates.

On the other hand the neurologist, studying recovery of sensory and motor ctions, will estimate that the rate of growth in the nerve is lower, since his teria depend on the presence of greater numbers of fibres, and on their medul- ion. If he studies the times of recovery of function in a certain muscle or part the skin after lesions at various levels he will obtain some estimate of the rate of vance of functional completion down the nerve trunk, and perhaps find it to be, in the rabbit, some 2 mm./day after suture, or 3 mm./day after crushing the ve. On the other hand if the rate of progress of sensory recovery in the skin, or times of recovery of muscles at various distances from the lesions are followed, rates may appear still lower. However, in man, with the greater distances ween muscles, the last-mentioned method should give good estimates of the rate growth in the nerve trunks.

In clinical studies there is likely to be the further complication that the apparent es of growth become lower as the functions studied become more complex. Thus nay be that only in those motor fibres responsible for 'simple' movements will ctional completion advance down the nerve at the 2·0 mm./day found after the ure for the simple functions of spreading of the toes in the rabbit. It would be eresting to know whether responses to, say, light touch, and particularly temperature, which recover later than pain, also advance more slowly. The work of Head & Sherren (1905) and Trotter & Davies (1909) clearly shows that there is a regular advance in recovery of all modalities of sensation.

With these qualifications in mind we may, finally, compare the above estimates of rates of regeneration with those found experimentally and clinically by previous workers. Few of these have taken account of the need to divide the process of recovery into its various periods. Vanlair (1894) did so in his pioneer paper on the subject, in which he speaks of the time (*d*) taken by proliferation and exodus of new fibres, *d'* the time in the scar, and δ that in the peripheral stump. He estimated *d* (by making, in dogs, two sutures in the sciatic on one side and one on the other!) at 40 days. *d'* was estimated, by removing 1 or 2 cm. of nerve altogether, as 0·25 mm./day, and δ, by a method very similar to that here used for the study of sensory recovery after high and low lesions, was found to be 1 mm./day. The latter estimate by what is at best a very difficult and ambiguous experiment, on two dogs, is apparently the foundation for many of the text-book statements that regeneration proceeds at this rate.

The most complete of later works is that of Cajal (1928). His method was the direct one of histological study. He recognized clearly that there is a delay before the fibres enter the scar, and that they then cross it slowly. Moreover, 'the sprouts are not equally vigorous and...the invasion of the foreign tissues occurs progressively as if by shifts'. He gives no details about the experiments he has done, but under conditions apparently comparable to our sutures the fibres take 'seven or more days' to reach the peripheral stump. Later however he says that 'usually one sees with certainty the axons in the peripheral stump...only from the 12th to the 15th day'. Our estimate of the scar delay for axon tips seems to lie within the limits of his observations.

Dustin (1917) used Tinel's sign to discover the length of the scar delay in man, and found that the sign began to be elicitable in the peripheral stump between 30 and 60 days after a good suture. Since this sign is usually held to depend on pressure on unmedullated fibres this seems a rather long delay. Huber (1920) considers that the regeneration is 'well under way by the end of the first week', and Lewis (1920) that 'neurofibrillae...grow down in the old sheaths by the fourteenth day'. Perroncito (1907) found new fibres in the peripheral stump after 6 days, even sooner in young animals.

It is clear from such results, and from our own, that axon tips begin to pass down the peripheral stump not more than about 10 days after a good suture. It is much more difficult to get any data from the literature about the time at which maturation to the level which we have called that of functional completion begins to advance. This delay would only be revealed by studies after lesions at various levels. Our results give 18 and 22 days for the degree of completion necessary to give return of response to pin prick in the foot and spreading of the toes respectively after crushing the nerve. After suture the data of p. 34 indicate that about 36 days elapse before the degree of functional completion necessary to give spreading of the toes begins to advance down the nerve. This is the figure which may be taken when nsidering the recovery of simple functions in man. Rates of advance of functional mpletion could then be calculated by subtraction of this number of days from the e taken for recovery, and dividing into the distance of the end-organ from the on

It has been assumed throughout this paper that the latent period recorded in eriments on recovery of sensory and motor function is the time necessary before portion of the nerve nearest to the lesion has matured to the state of functional mpletion. But there is the possibility, in the case of the experiments on motor overy (Fig. 5), that the delay before the earliest recoveries is partly taken up by process of making a functional contact with the muscle. If this were so then all lower points on Fig. 5 should be relatively far to the right, because they include extra delay, not present in the later recoveries. There are indications that this tor operates to a slight extent, and it is perhaps significant that the estimate of ent period from these motor recoveries (22 days) is somewhat greater than that m sensory recoveries (18 days), which, being based on distant lesions only, are affected by this factor.

Very few estimates of rates of growth in the peripheral stump make sufficient tinction between the various factors to allow strict comparisons. Cajal, again, has unambiguous estimate since he used histological methods. But he is aware of obscurities of the problem. 'Speed of growth of the nerve sprouts...is a problem which, owing to many disturbing causes difficult to measure, only a roughly proximate solution can be given.' He assumes that the 'vigour of growth, the a tergo of Held, declines progressively with time, until it becomes nil or almost ', and 'It would be very desirable to know the formula of this retardation'. e data of our Fig. 1 show no clear sign of such decline, though, as explained p. 18, they are not sufficient to show for certain that the growth rate remains nstant. After this very proper reservation Cajal then gives conclusions 'from merous measurements in rabbits, cats and dogs whose sciatic nerve was com-tely cut across'. The individual data are not given, but the conclusion is that 'The ocity of the cones of growth in the peripheral stump is between 2 and 3 mm. in hours....In some really exceptional cases the velocity of growth was 4 mm. per y'.

Huber (1920) states that 'the area of neurotisation progresses towards the riphery at the rate of 1–2 mm. per 24 hours', but he does not give the data on ich this figure is based, and it is certainly much too low.

Cajal's estimate agrees remarkably well with our estimate obtained from pinching exposed nerves, namely 3·5 mm./day after suture, and we may safely conclude t this is close to the rate of advance of axon tips in the rabbit and probably in the g and cat. The few estimates available indicate that the rate of advance of axon s is also similar in Man. Studying the advance of Tinel's sign several workers ve recorded figures much higher than the 1 mm./day of the text-books. Tinel mself (1917) gives 1–2 mm./day, but with no details, but Dustin (1917) finds es in the median nerve of 2·0–4·5 mm./day, and in the radial as high as 4–5 mm./ y.

Very few studies are available from which the rate of advance of functional completion of nerve fibres can be estimated. Osborne & Kilvington (1908), by the ingenious method of eliciting axon reflexes, found advances of motor fibres by as much as 140 mm. in 120 days (1·2 mm./day) and as little as 30 mm. in 91 days (0·3 mm./day). We have tried to use this method in the rabbit but though elegant in conception it does not give an estimate of rate of advance of recovery to a biologically significant level, and it proved very difficult to apply accurately in practice. That the recovery is quick if the lesion is nearer to the muscle is a commonplace of clinical studies but none have recorded the times or distances accurately enough to allow latent periods or rates of advance of functional completion to be studied accurately. Yet the data of Stopford (see M.R.C. Report, 1920) show that this process must advance much faster than 1 mm./day. For instance, m. pronator teres is said to recover in 4 months when the nerve is damaged at the elbow, 6 months when in the upper arm. The difference in average distance is not recorded but can hardly be less than 150 mm., which in 60 days give 2·5 mm./day.

The published data of clinical cases in man do not afford an accurate means of calculating the rate of advance of functional completion. They are mostly complex lesions (e.g. gunshot wounds) in which even after suture there may be fibrous scar tissue in the nerve and infection may further interfere with the process of regeneration. Moreover, secondary suture is performed after varying periods, and we do not know the extent to which this delay affects recovery. Also accurate measurements of the distance from the lesion to the end-organ have rarely been made clinically, and examinations are not made at strict and frequent intervals, so that an estimate of rate is extremely difficult.

Even studies of the rate of advance of recovery in the skin, which our studies show to give the lowest figures, usually show rates higher than 1 mm./day. Thus from the carefully recorded results of Trotter & Davies (1907) it can be calculated that in the skin the rates in mm./day are approximately 1·3 for touch (3480 mg.), 2·1 for cold sensation, 2·1 for pain and 1·2 for pilomotor function. These calculations assume that the distances travelled by the fibres are the same as those between points on the skin. Actually the distances are certainly greater and the rates therefore higher. The first return of sensation to the area shown in Fig. 14 of their paper occurred after 4 months (say 120 days) and the distance of the point of this recovery from the lesion was 200 mm. Subtracting 36 days for the delay before the process of functional completion advances this gives an estimate of growth rate of 2·4 mm./day, which is close to the rate which we have found in the rabbit.

We must conclude then, not only from our own results, but also from the published data, that the process of functional completion proceeds down the nerve faster than the usually accepted 1 mm./day, and that the axon tips grow faster still.

SUMMARY

1. Five different methods have been used to estimate the rate of advance of eneration along nerves in the rabbit. Not all give the same result. When the ance of the axon tips is studied a higher rate is recorded than when the times essary for functional recovery are considered. In the latter case the experiment es a measure of the rate of advance not of the tips of the axons but of completed ve fibres, able to function.

2. The 'scar delay', or period before arrival of new axon tips in the peripheral mp, is nearly constant in all well-made sutures (7·3 days). After interruption of erve by complete crushing at one spot this delay is only slightly shorter (5·2 days).

3. There is a further delay before maturation to the level of functional com- tion begins to advance down the nerve, so that the total latent period before ance of recovery begins is about 36 days after suture and 20 days after crushing erve.

4. The rate of advance of the fastest axon tips was determined by finding the thest point from the lesion at which reflexes could be elicited by pinching the ve. After suture of the n. peroneus in the rabbit this rate was 3·5 and after shing 4·4 mm./day.

5. This rate of the axon tips is not significantly different after crushing the ve at different distances from the nerve cell body, namely high in the thigh or ow the knee.

6. This rate is similar in the tibial, peroneal and sural divisions of the sciatic the rabbit.

7. The rate of advance of functional completion of nerve fibres was measured making lesions at various levels and studying (*a*) the time necessary for return response to nociceptive stimuli to a given point on the skin and (*b*) the time for overy of a given motor function, namely, spreading of the toes. Both methods e rates of 2·0 mm./day after suture, and 3·0 mm./day after crushing.

8. Analgesic areas on the foot shrink at the rate of 2·1 mm./day during recovery er crushing the nerve, 1·6 mm./day after suture.

9. Comparison of the times of recovery of muscles at varying distances from a ion gives an estimate of rate of advance of functional completion of 2·2 mm./day er crushing a nerve, but this method is only approximate in the rabbit.

10. In rabbits of 1 month old the rate of advance of the axon tips is not greatly ferent from that in adults, but the delay in the scar is less, and maturation of the ve fibres proceeds more rapidly.

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BLOOD SUGAR AND THE FOLLICLES OF LANGERHANS IN THE AMMOCOETE LARVA

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(With One Plate and One Text-figure)

I. INTRODUCTION

HE alimentary canal of the ammocoete larva is divided, posterior to the pharynx, to two regions, a very short, slender foregut (oesophagus) and a much wider idgut (intestine), the latter running back to the cloacal opening. At the point of nction of these two regions there are to be found, lying in the submucosa, a umber of small groups of cells which were first described by Langerhans (1873); ey have sometimes been referred to in the later literature as the 'follicles of angerhans', a term which, as the present work will show, is of some physiological well as historical appropriateness, and which might well be generally adopted. ojanus had earlier described a glandular mass in the corresponding region of the dult river lamprey; Rathke confirmed this, and Müller (1856) suggested that it ight be the pancreas, an interpretation which Langerhans seems to have accepted r the larval follicles on the ground that they were situated close to the opening the bile duct, which enters the alimentary canal at the extreme anterior end of the idgut. More recent workers, however, while continuing to regard these structures related in some way to the pancreas, have shown that their histological appearance nplies an endocrine function (Cotronei, 1923; Boenig, 1927, 1928, 1929), and this as led naturally to a revival of Brachet's (1897) suggestion that they may represent let tissue (Maskell, 1930; Barrington, 1936).

Now although comparatively little is known of the carbohydrate metabolism of e lower vertebrates, it is clear that the insulin mechanism is well established in em. Thus, the same types of islet cells as are found in mammals have been identi-ed in selachians (Thomas, 1940) and teleosts (Bowie, 1924). Moreover, shortly fter the original discovery of insulin, the hormone was shown to occur in both of ose groups (Macleod, 1922; McCormick, 1924; Vincent *et al.* 1925) and has since een extracted from the islets of the cod and pollock in a crystalline form identical certain properties with beef insulin (Jensen *et al.* 1929). Finally, the removal the pancreas from selachians, or of the separate 'principal islets' from teleosts, known to result, as in mammals, in a rise of blood sugar (McCormick & Macleod, 925; Simpson, 1926; Orias, 1932; Abramowitz *et al.* 1940), while insulin injections ave been found to produce hypoglycaemia or convulsions in some teleosts (Olmsted, 1924; Gray, 1928; Gray & Hall, 1930; Vorhauer, 1938). Taking these facts into consideration, it would seem reasonable to expect, on general evolutionary grounds, that a physiological mechanism so well established in fish might be detectable in the Cyclostomata, at least in a primitive if not in a fully developed form. It has therefore been the object of the present work to test the possibility of the follicles of Langerhans of the ammocoete being homologous with the islet tissue of higher vertebrates; for this purpose, attention has been confined to their histological structure, the effect upon them of the injection of glucose, and the effect upon the blood sugar of their destruction.

II. MATERIAL

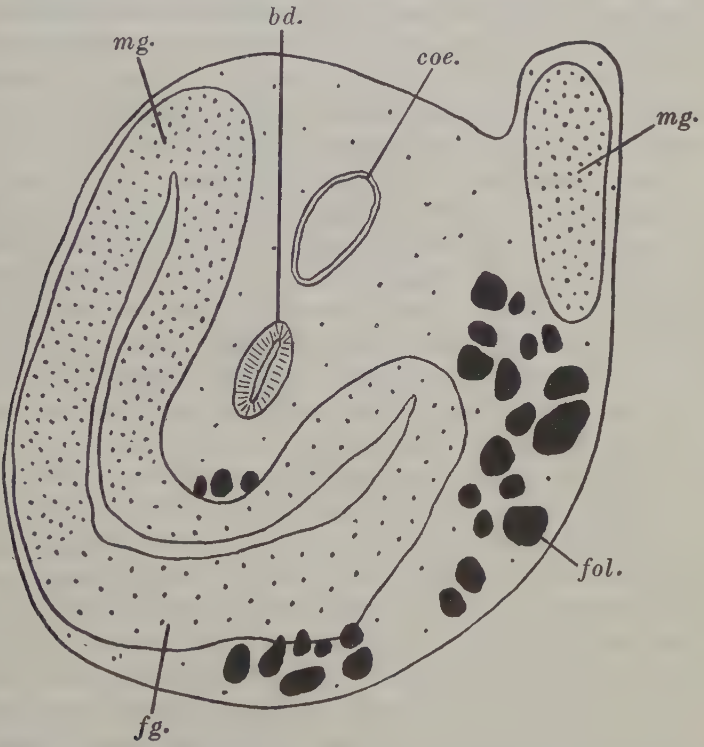
Preliminary work was carried out in England on ammocoetes of *Lampetra planeri*, but the results reported here were all obtained from animals taken from the region of Oneida Lake, New York State; they are assumed to be larvae of the land-locked lake lamprey, *Petromyzon marinus unicolor*, which is known to be common in the larger lakes of that district (Gage, 1928). The work was carried out in the spring and early summer on animals kept in a large indoor aquarium; this contained standing tap water which had been allowed to age over a period of twelve months while containing aquatic plants. In order to provide good oxygenation, the water was placed to a depth of only 1–2 in.; some green plants, *Gammarus*, *Daphnia* and a sprinkling of mud were added, and the temperature kept constant at 15° C. The animals, which were screened from the light, survived well during the limited period (not exceeding 2 weeks) in which they were kept in the laboratory.

III. HISTOLOGY

(*a*) *Normal*

Boenig (1929) has made a detailed study of the follicles using a variety of fixatives and stains, but since the publication of his work the use of Heidenhain's 'Azan' stain, following fixation in Helly's fluid, has become established as a particularly valuable method for the differentiation of the several types of islet cells both in mammals (Bloom, 1931; Thomas, 1937) and in selachians (Thomas, 1940), and it has therefore seemed worth while to re-examine the follicles from this point of view. For fixation and staining, the standard routine, as used by the above authors, was followed, sections being cut at $5\,\mu$.

The main facts established by Boenig, and essentially confirmed by the present work, are as follows. The follicles, which appear to develop throughout most of the life of the larva, mainly originate by budding from the epithelium of the foregut at the point where it passes into the midgut, although some also arise from the bile duct and from the extreme anterior end of the midgut itself. The general relationships in this region are shown semi-diagrammatically in transverse section in Text-fig. 1. The lateral tips of the midgut (*mg.*) project slightly forward alongside the foregut (*fg.*) and are thus cut in the same section as the latter, and at this point, as may be seen, the follicles (*fol.*) are conspicuous structures. All stages in their development may readily be traced from large nuclei visible in the basal region of epithelium; around each of these a granular cytoplasm forms, and division then duces a group of cells which pushes out into the submucosa. Here it separates pletely from the gut epithelium, and a central cavity develops (Pl. 1, fig. 2,). This cavity never communicates with the lumen of the gut, and thus any etion produced by the follicles can only be discharged into the blood stream. t such a secretory function is carried out is strongly suggested by their remark- extensive vascularization, for not only are they surrounded by blood channels, blood passes into their central cavities. This may be appreciated by reference to 1, fig. 1, which shows portions of two adjacent follicles (*fol.*); at the point *a* a



-fig. 1. Transverse section (semi-diagrammatic) through the alimentary canal of an ammocoete at the point of junction of foregut and midgut. *bd.* bile duct; *coe.* coeliac artery; *fg.* foregut; ollicles of Langerhans; *mg.* midgut.

d corpuscle lies in a blood channel between the two follicles, at *b* another one in a gap in the wall of one follicle, while other corpuscles (*c*) lie in the central ty of that follicle. In this way the distal ends of the follicle cells come to be ctly bathed by blood.

The application of Helly's fixative and the 'Azan' stain to mammalian islet e differentiates sharply between three types of cells, referred to as A, B, and D , the first of these being filled with fine, red granules, the second with orange- ones, and the third with blue-stained ones. Since these types have also been tified in selachians (Thomas, 1940) it is likely that they represent a widespread vertebrate characteristic, but two further types, the C cells found in the guinea-pi and the E cells found in the opossum, are at present of uncertain significance. Th existence of granules in the ammocoete follicle cells is well established, but som workers claim to have identified, both in the larva and adult, two types of cells dis tinguished by their staining reactions as 'light' and 'dark' (Giacomini, 1900 Cotronei, 1923, 1927). Boenig (1929), however, concluded that in the larva thes were merely two functional stages of the same cell type, and this is confirmed by th present work. The 'Azan' technique reveals in the cells a number of blue-staine granules set against an orange or neutral-tinted background, these granules bein visible even before the follicles have separated from the gut epithelium. No othe staining reaction has been identified, and in particular no red-stained granule which would be characteristic of A cells, have been seen. There is thus som difference between the follicles and typical islet tissue, and it is even uncertai whether the cells of the former can be compared with any of the types describe above. As far as the presence of blue-stained granules is concerned, the closes resemblance is to the D cells, but whether or not this is of significance cannot b decided until more is known of the function of the latter. In the human pancrea Bloom (1931) found some cells suggestive of a transition between D and A, an between D and B, but more recently Gomori (1941) has considered D cells to b degenerating A cells, and if this latter view is confirmed there can be little profit i comparing the ammocoete cells with them. It would in any case be unwise t press such a comparison too far, for the mere fact of staining with aniline blue i not in itself a specific reaction.

That the follicle cells are secretory structures seems clear, for their contents ar discharged into the central cavity and thus into the blood stream. According t Boenig (1929), the secretory mechanism is of the holocrine type, and it is true tha nuclei can sometimes be seen lying in the cavity, but whether a loss of nuclei alway accompanies a discharge of secretion is not clear; the experiments with glucose, t be described below, suggest that they may sometimes persist.

It may be concluded from the above that while the structure of the follicles o Langerhans implies that they have an endocrine function, their staining reaction do not demonstrate any decisive resemblance to the islet tissue of higher forms; fo the obtaining of more definite conclusions, reliance must evidently be placed on a experimental approach to the problem, and such will now be described.

(*b*) *The effect of glucose injections*

It is now commonly accepted that the B cells of the islets are the source o insulin, this belief resting in part upon the fact that injections of glucose have bee shown to result in degranulation and hydropic degeneration of those cells, such a might be associated with hypersecretion of the hormone in response to the presenc of excess sugar. Thus Gomori *et al.* (1939), injecting 2 g./kg. of glucose in 10% solution into guinea-pigs, produced a rise of blood sugar which was maximal a 3 hr. after the injection, and found that the B cells of all animals exhibiting thi hyperglycaemia showed a reaction ranging from a sparsity of granules to almos mplete absence of them, while the A cells were unaltered. In the light of such ults it has seemed desirable to examine the effect of glucose injections upon the mocoete follicles, again using Helly's fixative and the 'Azan' stain. Animals ging in weight from 1 to 3 g. were used, and were given intraperitoneal or sub- thelial injections of glucose dissolved in frog Ringer or in distilled water to a ength of 10 or 20%. Some animals received a single injection of 0·05 or 0·1 c.c., d were killed after 24 hr., while others received up to four successive ones of ·5 c.c. each at 12 hr. intervals, and were killed 12 hr. after the last dose; the ct quantity of glucose received by the animals is naturally unknown, since some the fluid escapes from the point of injection. Thirteen animals were treated in s way, and a further nine received control injections of corresponding volumes of tilled water or Ringer.

Follicles from the control animals, one of which is shown in Pl. 1, fig. 2, re- mbled normal, untreated ones in the homogeneous granulation of their cells, but nditions were quite otherwise in the majority of those which had received glucose. these, nine showed a marked response, two others a slight one, while the re- ining two showed none. This response involved a conspicuous breaking down d vacuolization of the cells, the exact appearance varying in different follicles and different animals. In some the follicles appeared solid, with vacuoles distributed ough them; in others the distal region of the cells had broken down, so that the ntre of the follicles were filled with an ill-defined cytoplasmic reticulum; in ers again, groups of cells were seen in which the whole of the region above the cleus was empty. Not all of the cells in a follicle were equally affected, granules ing still visible in many of them. The development of vacuoles, which has never en observed in the control animals, is perhaps the most striking of the changes ted, and is illustrated in Pl. 1, fig. 3 (*vac.*); whether it is to be regarded as identical th the hydropic degeneration recorded in B cells under similar conditions is not ar, but it certainly implies that the injection of the glucose has induced hyper- cretion. This, it may be noted, has been said to be the essential characteristic of dropic degeneration; in its first stage it is not a degenerative change, but merely a cuolization and functional exhaustion which only later becomes carried to the int of cellular destruction (Allen, 1922; Martin, 1922).

Mention has been made above of the view that the normal follicles seem to be mposed of only one type of cell, and this interpretation is supported by the pearance of the cells in the glucose-injected animals, for while the responses of e individual cells vary, the differences are merely quantitative, there being no nstant differentiation of a negatively responding cell type, such as is provided by e A cell in the islet tissue of mammals (Allen, 1922). In connexion with Boenig's 929) view that secretion in the follicles is holocrine and is accompanied by a distal ovement and discharge of the nuclei, it is to be noted that most of the nuclei ain their normal basal position during the reaction to glucose; however, in the esent work the animals have not been kept long enough to show whether the cells e capable of regeneration after this sudden demand, or whether the nuclei are entually lost.

Evidently this response of the follicles, while in no sense a proof of the secretio of insulin in the ammocoete, gives support to the view that they are involved i some way in the regulation of carbohydrate metabolism.

IV. BLOOD SUGAR

(*a*) *Methods*

For measurement of the blood sugar, each animal was first anaesthetized b immersion in a 1 % solution of urethane. The body surface was then wiped dry, little powdered potassium oxalate applied immediately behind the last gill openin and a ventral incision made with scissors into the heart in that region. It was the possible to collect a drop of blood in an oxalated watch-glass, and to take up 0·02 c.c into a blood pipette, the estimation being made by the method of Hagedorn an Jensen, modified by Rappaport & Pistiner (1934) for that volume. Glucose solution of known strength could be estimated by this method with a mean error of 11 % and glucose added to blood samples could be recovered quantitatively within th same limit. Owing to the small size of the animals and the rapid clotting of th blood, it was usually possible to secure only one sample from each, but from seve animals two samples were obtained, and the paired results were found to show mean difference of only 9 mg. %. It should be borne in mind that the object o this study has been to establish a difference between the blood-sugar values of tw groups of operated animals, and it will be seen that, although the above errors ar larger than would be expected with the original method of Hagedorn and Jensen they do not disturb the significance of the results to be reported. The collection o the blood by drainage introduces some possibility of contamination with wate from the pharynx; this, however, can be largely eliminated by careful squeezing o the pharynx before making the incision, and any residual error can be neglected since it would be distributed equally between the two groups of animals.

For simulating pancreatectomy, it was necessary to destroy the follicles rathe than to remove them. This was effected by anaesthetizing the animal, opening th anterior end of the body cavity out of water, cauterizing the intestinal wall in th region of the follicles with a hot needle, and then replacing the intestine and closin the wound with a single suture. Control animals were treated similarly, except tha in some a small portion of the middle region of the intestine was cauterized, whil the remainder had no cauterization at all. No difference was noted between th blood-sugar values of the two types of control animals, which are therefore treate in Table 2 as one group. The operated animals were transferred to wooden tub containing a shallow depth of the same water as that used for the main stock, and like the latter, were kept at a temperature of 15° C.; they survived well during th period (up to 3 days) for which they were required to be maintained alive.

(*b*) *The normal blood sugar*

For comparison with the operated animals, the blood sugar of eleven normal unoperated ammocoetes was measured; the results are recorded in Table 1 an show a mean value of 90 mg. %. No previous work on the ammocoete is to b d, but White (1928) obtained values of 64–68 mg. % for the mixed blood of t adult lampreys (*Entosphenus tridentatus*), and McCay (1931) values of mg. % for the blood of four adults in prime condition at spawning, and g. % for four exhausted after spawning. Values obtained by different authors ish range over a wide scale, from 20 mg. % for *Torpedo ocellata* (Gray & Hall,), for example, to 160 mg. % for the eel (Kiermeir, 1939).

Table 1. *The blood sugar of normal, unoperated ammocoetes. Here and in Table 2 the blood sugar is given in mg. % to the nearest 5 mg., and standard errors to the nearest unit*

Length of animal cm.	Blood sugar	Length of animal cm.	Blood sugar
13·5	120	11·5	50
13	60	11	75
12·5	90	11	55
12·5	55	10·5	135
12	105	10·5	115
11·5	90	10·5	110
Mean 90 ± 8			

As may be seen, the individual variation bears no relation to the length of the nals; how far it is due to experimental error, as discussed in the preceding ion, and how far it is normal, is not clear, but various authors have reported iderable individual variation amongst fish; Vorhauer (1938) and Kiermeir 9), for example, give values for *Tinca vulgaris* ranging from 50 to 200 and 60 to mg. % respectively. The situation is complicated by the possibility of the urrence of 'alimentary hyperglycaemia' which may persist for days after feeding nten, 1927), since food remains for a long time in the stomach of the lower ebrates (Barrington, 1942); in this connexion it is significant that Abramowitz *et* 1940) found that the standard error of the normal blood-sugar values of *Mustelus* *s* was high after feeding, but became low after the second day of starvation. In present work no attempt was made to maintain the ammocoetes under normal ing conditions, but they could doubtless have obtained some nutriment from the nic material in the water.

(*c*) *The effect of cauterization of the follicles of Langerhans*

Table 2 gives the blood-sugar values of thirty-two follicle-cauterized and the e number of control-operated animals, killed at varying periods after the ation. It will be seen that after 48 and 72 hr. there is a reaction similar to that luced by pancreatectomy in the other vertebrate groups, for there is a marked erglycaemia in the follicle-cauterized forms, their blood sugar being respectively nd 93 % above that of the corresponding controls. Comparison of the standard r of the means shows these differences to be highly significant; for the 48 hr. p, $d/\sigma = 4{\cdot}3$, and for the 72 hr. group, using the method for small samples,

$t = 4{\cdot}5$ (Simson & Roe, 1939). At 24 hr. the difference is much smaller and statistically is of doubtful significance, but it may be inferred that hyperglycaemia i gradually becoming established during the first day.

It is not certain whether any special significance can be attached to the smal variations in the mean blood sugar of the three control groups, but it should b understood that the incision in the operated animals could not be completel sealed, so that the internal organs were to some extent in contact with the outsid water; this might be expected to result in osmotic disturbance of the compositio of the blood, and the 72 hr. group does offer some suggestion of a fall in the bloo sugar of the controls, but the difference is not decisive enough to be significant. A regards the range of individual variation in the follicle-cauterized forms, the sam considerations apply as have been discussed above in connexion with the unoperated animals, but there is also the probability that not all the animals can be expected t react to the same extent. This is certainly true of *Mustelus canis*, in which species there is great variation in the response to pancreatectomy, only 56 % of the operated animals showing severe diabetes, the remainder ranging from a condition of mild diabetes to actual hypoglycaemia (Abramowitz *et al.* 1940).

Table 2. *The effect of cauterization of the follicles of Langerhans upon the blood sugar of ammocoetes*

Interval between cauterization and drawing of blood					
24 hr.		48 hr.		72 hr.	
Follicles cauterized	Controls	Follicles cauterized	Controls	Follicles cauterized	Controls
150	150	225	110	170	105
170	125	220	115	195	75
105	105	200	85	75	55
125	115	170	160	170	85
95	95	195	110	175	85
135	100	140	105	130	80
110	85	120	85	95	45
100	65	115	70	150	70
100	115	140	100	100	60
		95	110	200	
		130	40		
		95	95		
		150	65		
			60		
120 ± 8	105 ± 8	155 ± 12	95 ± 8	145 ± 13	75 ± 6

It should be added that Table 2 comprises the results of a continuous series of experiments, previous to which the procedure had been standardized in an extensive preliminary study, but to counteract further any possible day-to-day variation in technique, control animals were always handled simultaneously with follicle-cauterized ones, three of each being usually prepared. These sets of animals are separated by spaces in the table, and it may be seen that the blood sugar of each cle-cauterized group is consistently higher than that of its corresponding control ıp, at least as far as the 48 and 72 hr. forms are concerned.

V. DISCUSSION

In recent years it has become clear that the control of carbohydrate metabolism he mammal is a function of great complexity, in which at least five internal etions appear to be antagonistic to insulin (Best & Taylor, 1939), and it would efore be premature to generalize from the present work as to the situation in the ocoete. Nevertheless, in the reaction of the follicles to glucose, and in the erglycaemia induced by their destruction, there is a definite similarity to the wn properties of the B cells of mammalian islet tissue, and an implication of the ibility of the secretion of insulin, although it may be doubted whether the ence of the hormone could be established without the use of an extravagant ber of animals. Maskell (1930) reported an unsuccessful attempt to extract in from six adults, and it is hoped to repeat this work on a larger scale.

The fact that the follicle cells are of only one type, and that this does not ly resemble the B cells of typical islet tissue, need not occasion surprise, for it be remembered that the Cyclostomes are an exceedingly primitive group, rently separated from modern fish by an extinct group of intermediate organiza- the Aphetohyoidea (Watson, 1937), and it may well be that at such an early of vertebrate evolution the islets would be represented by a primitive and ively undifferentiated tissue. From this point of view, and having in mind the ence in the follicle cells of blue-staining granules, it would be of great interest D cells of islet tissue proved to be in some way related to the development of lls, but, as mentioned earlier, the only evidence at present available does not ort such a possibility. Further, it should not be overlooked that the ammocoete developing larva, and a comparison of the follicles should more properly be e with the developing islets of the higher vertebrates rather than with the fully olished tissue; here again, however, there is as yet no adequate information which such a comparison could be based. It may be added that staining ions unsupported by other lines of evidence provide an unreliable basis for parisons. For example, it is a common experience in staining secretory cells he 'Azan' method to find that the same inclusion may take a blue or an orange ur according to its state of development. Thus the positive reaction of the cle cells to glucose outweighs in importance the actual staining reactions of cells.

The significance of the present work for an understanding of the early history e vertebrate pancreas will be discussed in later publications dealing with adult ostomata. It will be sufficient to state here that it goes some way to confirm view (Brachet, 1897; Maskell, 1930; Barrington, 1936) that in the ammocoete the two components of that organ are identifiable but not yet intimately ciated, the zymogen cells being still confined to the lining epithelium of the gut, with some concentration at the anterior end, while the homologue of the islet tissue is developing in their close vicinity. It may be suggested, in conclusion, that the designation of the follicles as 'follicles of Langerhans' (Maskell, 1930) might well come into general use, since it aptly expresses both the name of their discoverer and their apparent involvement in carbohydrate metabolism; the establishment of their exact relationship to the islets of Langerhans must await further investigation.

VI. SUMMARY

Glandular follicles, first described by Langerhans, develop in the wall of the alimentary canal of the ammocoete larva at the point of junction of fore- and midgut, and have often been compared with the pancreas of higher forms. They have been re-examined histologically, using Helly's fixative and the 'Azan' stain, and the view of some recent workers, that they have the appearance of endocrine structures, is confirmed. They comprise only one type of cell, however, which shows no decisive resemblance in staining reaction to any of the cell types characteristic of typical islet tissue.

The injection of glucose into ammocoetes usually produces a marked response in these cells, involving extensive vacuolization, while destruction of the follicles by cautery induces a significant rise in blood sugar. These reactions, which imply that the follicles are involved in carbohydrate metabolism, are similar to those associated with the B cells of islet tissue.

It is concluded that the evidence, while not yet complete, suggests that the follicles represent a primitive stage in the evolution of the endocrine component of the vertebrate pancreas, and it is pointed out that 'follicles of Langerhans' would be an apt designation for them. Further work will be necessary to clarify their relationship to the islets of Langerhans of higher forms.

This work was completed during the tenure of a Rockefeller Foundation Fellowship. It is a pleasure to acknowledge the facilities and co-operation enjoyed in the Departments of Physiology and Zoology, McGill University, and the Biological Laboratories, Harvard University.

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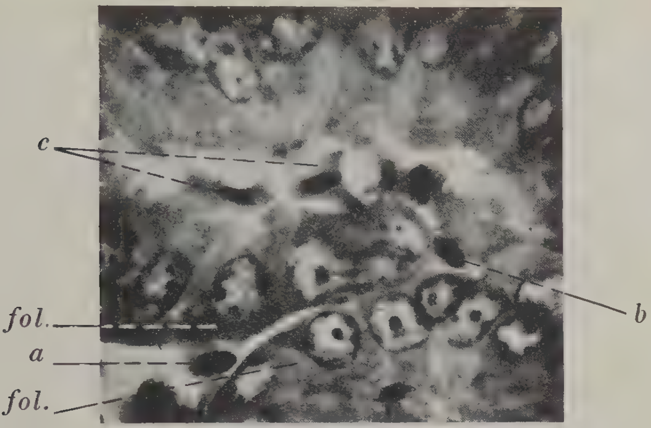

Fig. 1

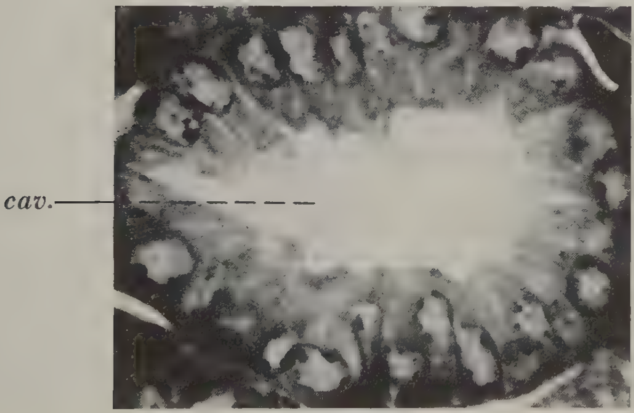

Fig. 2

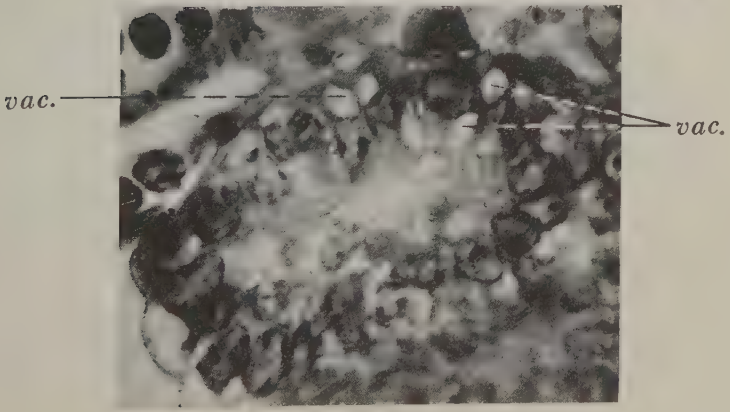

Fig. 3

ARRINGTON—BLOOD SUGAR AND THE FOLLICLES OF LANGERHANS IN THE AMMOCOETE LARVA (pp. 45–55)

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EXPLANATION OF PLATE 1

verse sections (5 μ) through the follicles of Langerhans of ammocoete larvae; Helly's fixative, stain. Photographs taken with a Leitz $\frac{1}{12}$ in. oil-immersion objective and Zeiss Homal ocular, and reproduced at a magnification of × 1200.

Normal unoperated ammocoete.

Ammocoete killed 24 hr. after receiving an injection of 0·1 c.c. of distilled water.

Ammocoete killed 12 hr. after receiving the last of four injections of 0·05 c.c. of 20 % glucose, at 12 hr. intervals. *a*, *b*, *c* blood corpuscles (see text); *cav.* central cavity of follicle; *fol.* wall icle; *vac.* intracellular vacuole.

THE STORAGE OF PROTEIN, FAT, GLYCOGEN AND URIC ACID IN THE FAT BODY AND OTHER TISSUES OF MOSQUITO LARVAE

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(With Eleven Text-figures)

It is generally agreed that the main function of the fat body of insects is the storage of those reserve substances which require rapid mobilization during moulting and metamorphosis. But little attention has been given to the part taken by other organs and tissues in building up reserves and almost none to the influence of the composition of the food upon the type of reserve substance laid down.

These problems form the subject of the present paper. Mosquito larvae have been starved until all their reserves have been consumed. They have then been fed upon single substances and the storage of protein, fat and glycogen in the various tissues observed.

METHODS

Rearing and starving of larvae

All the experiments have been made at 28° C. on larvae of *Aedes aegypti* in their fourth or final instar. The eggs are immersed in a rich infusion of powdered dog biscuit. They hatch at once and the larvae reach the 4th stage in about 48 hr. If they are left in the infusion they pupate in a further 48 hr.

The larvae are starved by transferring them, within an hour of moulting to the 4th stage, to freshly prepared 0·01 *M* acid potassium phthalate in tap water adjusted to *p*H 4·0 with phosphoric acid. They are kept in this at 28° C. until their reserves are used up. This requires about 10–14 days. The fat body of the living larva can be observed by transmitted light under the microscope. By the time all fat droplets have disappeared the reserves are practically exhausted (p. 62). The larva then becomes sluggish and generally dies within 48 hr. or so.

Feeding of larvae

After this preliminary starvation the larvae are given pure food substances, twelve larvae being placed in 2·5–5 c.c. of the phthalate buffer at *p*H 4·0.

Protein is given in the form of casein (fat free and vitamin free). This is finely ground with kaolin and a few milligrams added on the point of a knife.

Carbohydrates. Starch is given in the same way in the form of soluble starch ground with kaolin and washed sand. *Sugars* are given in 2·5 % solution with addition of kaolin. The larvae ingest the kaolin and so obtain the solution with h it is moistened. Starved larvae immersed in such a mixture have the entire filled with kaolin within about 2 hr.

at is given in the form of olive oil. A few drops of olive oil containing a drop eic acid were emulsified with dilute sodium carbonate to form a stable emulsion out the density of milk. Two or three drops of this are added to 5 c.c. of the alate buffer containing a large knife point of kaolin. The oil droplets are en-ed by the kaolin and held at the bottom of the fluid where they are ingested he larvae. Lecithin emulsified in water was given in the same way.

Exclusion of bacteria

This has been effected by keeping the larvae at the acid reaction of pH 4·0 and ansferring them to freshly prepared food mixture every 12 or 24 hr. It is not ned that these cultures are sterile in the bacteriological sense. But in a number sts in which casein mixtures (these are the most liable to contamination) were unchanged after removal of the larvae, hanging drop preparations showed that o-organisms were still very few at the end of 2 or 3 days. They are therefore ely to be of nutritional importance during the first 12 hr. of growth.

This belief is supported by the fact that although, as will be seen, casein is an uate source of protein, fat and glycogen, larvae fed with casein under these itions may still show no sign of growth beginning in the imaginal disks, etc., e end of 7 days; whereas larvae fed with casein containing abundant micro-nisms (and so providing the accessory factors needed for growth; cf. Trager, , 1937) will pupate within 4 days.

Nevertheless, yeasts which grow rather rapidly at pH 4·0 have occasionally ared in the cultures. These have therefore been regularly examined in hanging preparations and the experiment rejected when such organisms were present.

Examination of the tissues

i) Many observations on the fat body can be made in the living insect. Below cuticle of the dorsal and ventral surfaces of the larva the flattened cells of the ody form a thin single layer. In each cell the various inclusions lie for the most in one plane and may be observed with the 2 mm. objective. It is thus possible llow the inclusions in a given cell from day to day or from hour to hour, and at desired stage to fix and test them. The distribution of glycogen in these cells e seen by immersing the living larva in 0·05 % iodine solution under a sup-ed cover-slip.

ii) The general distribution of reserve substances is best studied in whole e dissected. After fixation the neck and the last abdominal segment are cut ugh. The larva is then slit with scissors along one side and the gut removed ugh the slit. The body may now be unrolled and mounted flat and all the es (epidermis with imaginal disks, fat body, tracheae, heart and pericardial muscle fibres and sarcoplasm, oenocytes, reproductive and nervous systems) e examined with the oil immersion in a single preparation (Fig. 1). The gut caeca and Malpighian tubes is best cut longitudinally and mounted flat along-

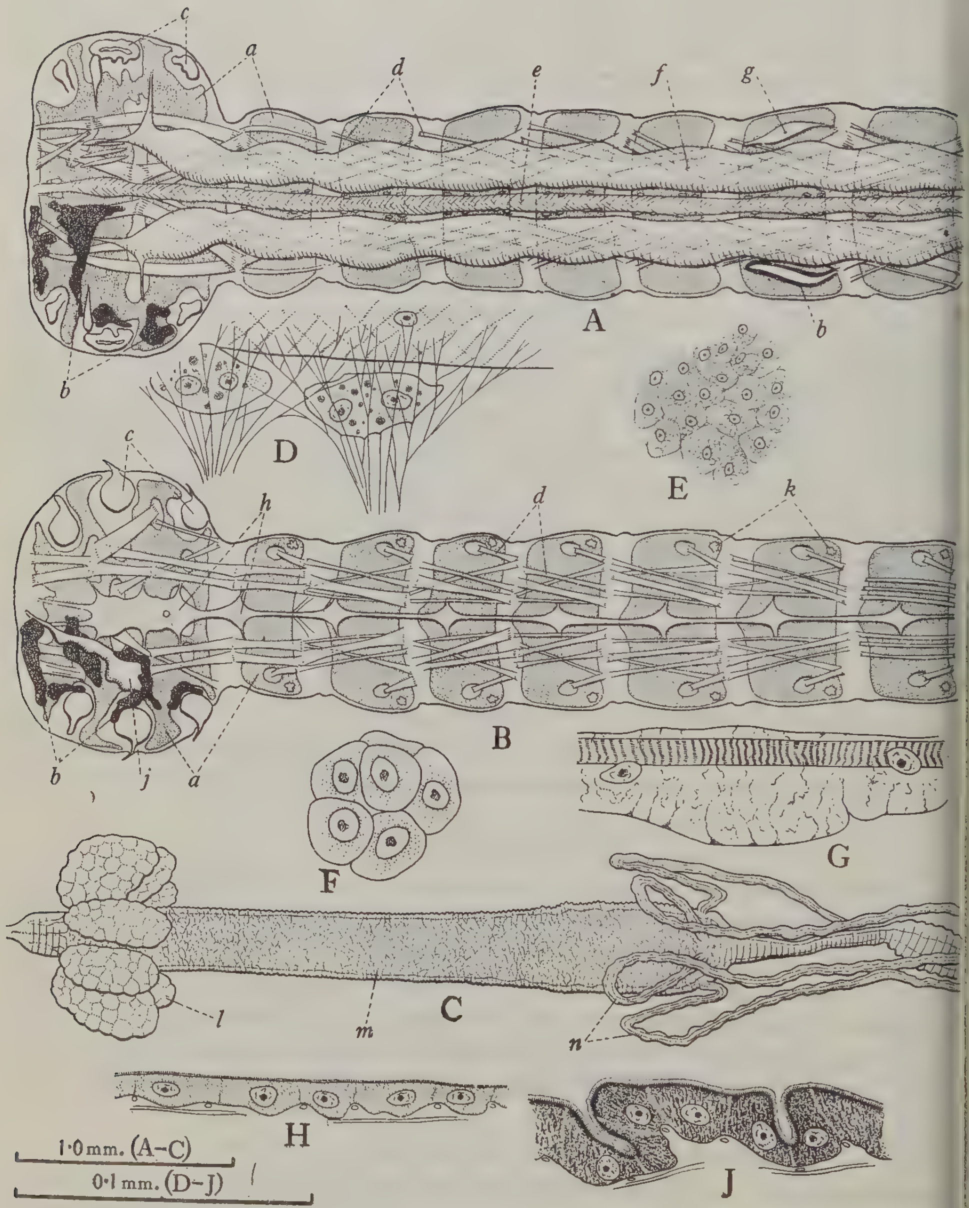

Fig. 1. Anatomy of mosquito larva showing the chief tissues used for storage. A, dorsal half o larva; B, ventral half of larva; C, alimentary canal; D, pericardial cells and margin of heart; E, fa body; F, oenocytes; G, muscle with its sarcoplasm; H, longitudinal section of anterior half of midgut; J, section of posterior half of midgut. *a*, parietal fat body; *b*, visceral fat body; *c*, imaginal disks *d*, muscles; *e*, heart with pericardial cells; *f*, main tracheal trunk; *g*, gonad; *h*, ventral nerve cord *j*, salivary gland; *k*, oenocytes; *l*, gastric caeca; *m*, midgut; *n*, Malpighian tubes.

the remainder of the body after removal of the peritrophic membrane and its ents.

The following tests for the chief reserve substances have been used.

Protein. The larvae are fixed in Carnoy's or Bouin's mixtures and after dis-ion are warmed in 50% glycerol containing 1% of ninhydrin or in Millon's ent freshly mixed with an equal volume of glycerol. The violet colour of the eins in the ninhydrin and the rose red in Millon's reagent are permanent in arations mounted in glycerol or in syrup.

Fat. The larvae are fixed in Bouin's fluid, stained with red or black Sudan B mounted in glycerol. A few larvae have been fixed in the dilute Altmann's (equal parts of 3% potassium dichromate, 2% osmic acid and water) recom-ded by Baker (1933). This gives excellent fixation of the cells, but the blackening e inclusions is by no means specific for fats (Lison, 1936).

Glycogen. The larvae are fixed in Carnoy's fluid or in alcoholic Bouin. They are stained with light green in 95% alcohol and then in a mixture of absolute hol saturated with iodine (65 parts) and 1% iodine in potassium iodide (35 parts), drated in 95% alcohol saturated with iodine and mounted in euparal saturated iodine. In these permanent preparations the glycogen deposits are conspicuously ed; but they are somewhat shrunken and distorted. Their natural form is better erved if the larvae are mounted direct from the 65% alcoholic iodine into olene oil'.[1] Unfortunately these preparations are not permanent; the glycogen ns to fade after 24 hr.

n order to demonstrate fat, glycogen and protein in a single cell, the larva is for 12 hr. in Bouin's mixture made up with 85% alcohol saturated with picric It is then stained with B.Z.L. blue (Ciba) in 60% alcohol, mounted in syrup the distribution of fat in the selected cell drawn. The fat stain is removed in alcohol, the glycogen is stained in 65% alcoholic iodine, the larva mounted in olene oil' and the cell redrawn. Finally, the iodine is removed in tap water and ein is stained with ninhydrin. Figs. 6, 8 and 10 were obtained in this way.

iii) Histological details have been confirmed in sections of larvae double edded in celloidin and wax and stained with haematoxylin and eosin. The tests rotein and glycogen have also been applied to such sections.

RESULTS

Anatomy and histology of the larva

The general arrangement of the tissues and the histology of those used largely torage are shown in Fig. 1. The *fat body* consists of a parietal layer composed single sheet of flattened cells applied to the surface of the body in each segment, a visceral layer made up of a few small lobes in the prothorax, groups of cells ied to the imaginal disks and a thin membrane covering the gonads. The dorsal ody is more deeply pigmented (it contains more uric acid) than the ventral; wise the structure is fairly uniform throughout.

The *gut* is shown in Fig. 1 C. Immediately behind the midgut caeca there are a few rather darkly staining cells, but beyond these the midgut falls into two sharply divided halves. In the anterior half (Fig. 1 H) the cells are clear, pale staining and homogeneous; in the posterior half (Fig. 1 J) they are granular and deeply staining with a conspicuous striated border. This division seems to be widespread in larvae of Nematocera. It is described among others by van Gehuchten (1890) in *Ptychoptera*, by Frederici (1922) in *Anopheles* and by Samtleben (1929) in *Culex*.

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[1] A wetting and clearing agent, miscible with alcohol and water, marketed by Imperial Chemical tries, Ltd.

Reserve substances in the newly moulted 4th stage larva

In larvae newly moulted to the 4th stage *fat* droplets of varying size are plentiful throughout the fat body; there may be a small amount of fat in the cells of the anterior half of the midgut and there is a diffuse staining which cannot be resolved into droplets in the central fibrous region of the nerve ganglia, but no stainable fat elsewhere.

Protein in the fat body consists only of a condensed zone around the nucleus with filaments radiating outwards between the fat droplets and other vacuoles; there are no discrete deposits of protein. Elsewhere, also, protein exists only in the form of cytoplasm; it is most dense in the muscle fibres, oenocytes, pericardial cells and in the posterior half of the midgut.

Glycogen is widely dispersed. It is plentiful in the fat body (Fig. 2 A) and it occurs throughout the ganglia and nerve cord of the central nervous system in the form of granular deposits and small vacuoles (Fig. 2 C). But it is most abundant in the muscles, where the swollen masses of sarcoplasm which surround the fibres are practically solid with glycogen. There are also thread-like deposits between the muscle fibres (Fig. 2 B). Smaller amounts occur on the fibres of the gut wall and of the heart and there are small deposits in the intestinal epithelium, particularly in the caeca and the posterior half of the midgut. Glycogen is absent from the oenocytes and pericardial cells.

Fig. 4 A shows two typical fat body cells as seen in the living larva newly moulted. The cytoplasm appears as a coarse meshwork enclosing large vacuoles, and the cell boundaries are not visible. The vacuoles are of two types, one containing refractile droplets of fat, the other with non-refractile aqueous contents. The latter are mostly spherical but some are elongated or irregular in outline. Their interfacial tension is low, for these vacuoles are deformed and their contents move to and fro as the cells are pressed by the contracting heart. They range in size from minute vacuoles only a few μ in diameter to large spaces several times the diameter of the nucleus. They may be colourless, pale yellow or grey. They are responsible for the range in colour of the larva from white to dark grey.

After fixation (Fig. 2 A) some of these watery vacuoles, particularly around the periphery of the cells are seen to contain glycogen. But most of them contain none of the reserve substances.

If the larva is fixed in aqueous Bouin, dissected in 90% alcohol, and mounted in euparal (Fig. 4 A′) it is seen that in many of the vacuoles small sheaves of needle-like crystals of uric acid[1] have separated out.

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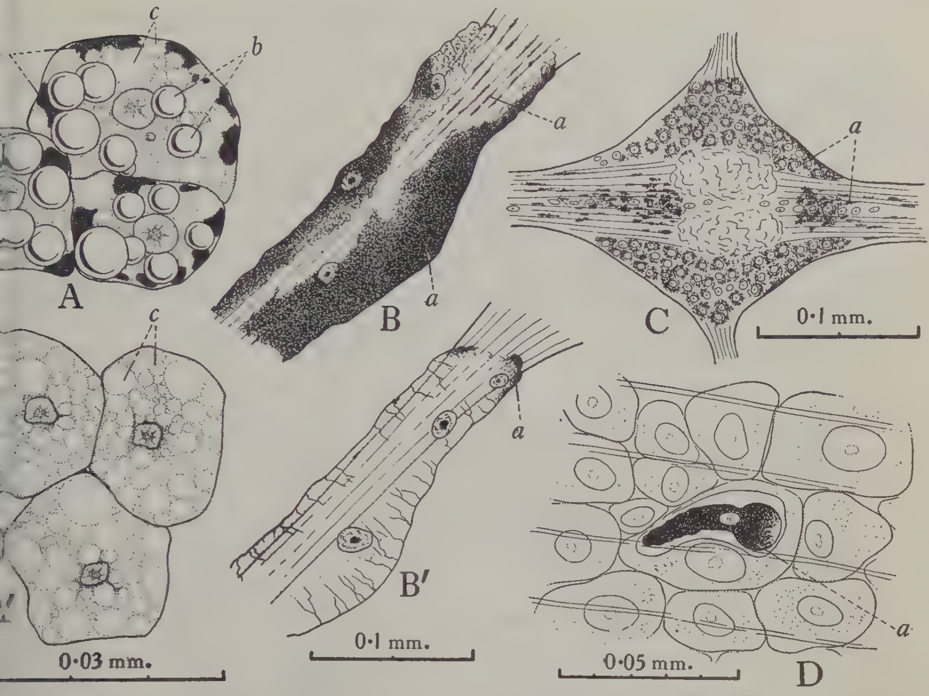
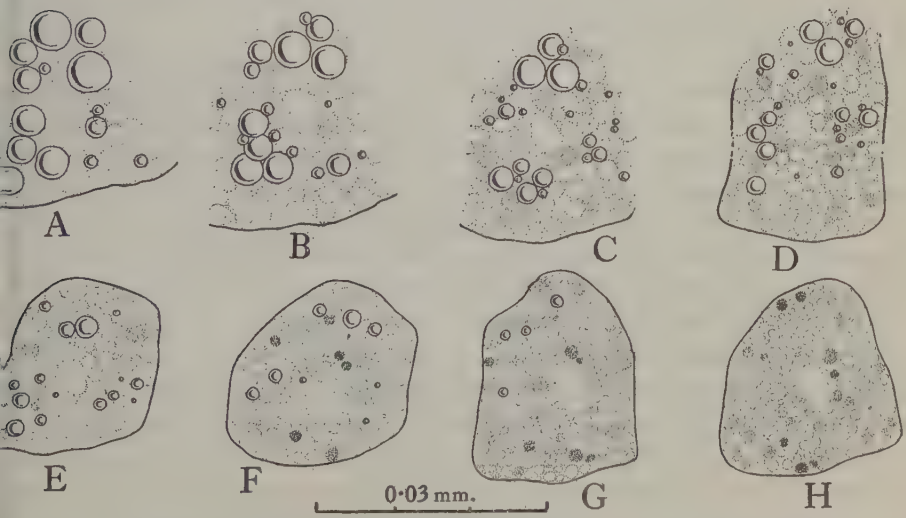
[1] These crystals are assumed to be uric acid because they are soluble in dilute alkalis, and in aqueous solutions of piperazine and hexamine; they give a positive murexide reaction and a blue colour with Folin's uric acid reagent (cf. de Boissezon, 1930).



A, fat body cells of newly moulted fourth stage larva; A′, the same in larva starved 12 days; of muscle and sarcoplasm of newly moulted larva; B′, the same in larva starved 12 days; ominal ganglion in newly moulted larva; D, surface view of midgut wall in completely larva, showing *Lankesteria culicis* in one of the cells. *a*, glycogen stained with iodine; *b*, fat ts; *c*, watery vacuoles.



. A single fat body cell at the hind margin of the metathorax drawn from the living larva. A, immediately after moulting to the fourth stage; B–H, at 12 hr. intervals thereafter.

Utilization of reserve substances during starvation

Fat, protein and glycogen are used concurrently throughout starvation. There no indication that glycogen is used up first. Fig. 3, for example, shows the f droplets in a fat body cell drawn at intervals of 12 hr. during the first 3 days starvation. The fat droplets move rather freely about the cell and are progressive reduced in size, minute droplets being apparently detached from the larger ones.

The fat droplets disappear first in the abdomen; they persist longest in the pro thorax, where they finally disappear from 10 to 14 days after moulting. The larv dies about 48 hr. after this. Even in larvae completely starved, fat droplets remai at the centre of the disks of the pupal respiratory trumpets.

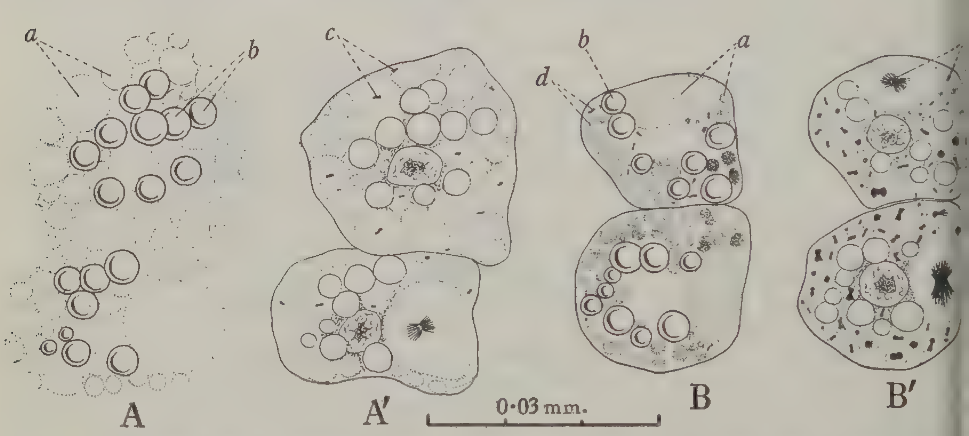


Fig. 4. A, fat body cells in thorax of newly moulted living larva; A′, the same cells after fixation aqueous Bouin; B, fat body cells in thorax of living larva starved 7 days; B′, the same cells aft fixation in aqueous Bouin. *a*, watery vacuoles; *b*, fat droplets; *c*, uric acid crystallized after fixatio *d*, uric acid separated in vacuoles of living cell.

The disappearance of glycogen from the fat body runs parallel with that of fa The amount is much reduced at the end of 4 days; none remains after 6 day (Fig. 2 A′). At the same time the deposits in the gut have disappeared. In th sarcoplasm of the muscles the deposits are becoming less massive and solid by th end of 6 days; by 10 days they are very slender in most places; at 12 days, in larva from which all fat had been used up, there were still faint traces of glycoge particularly around the points of insertion of the muscles (Fig. 2 B′). It is o interest to note that when such larvae harboured gregarines (*Lankesteria culicis*) i the gut, these were still stuffed with glycogen (Fig. 2 D).

Alongside the utilization of fat and glycogen there is a progressive wastage o cytoplasm in all the tissues as proteins are used up, and the nuclei become muc reduced in size. This is well seen in the muscle fibres and in the fat body cells. Th oenocytes, pericardial cells, gut and central nervous system show a similar wastage.

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[1] Within the cells of the midgut, particularly in the caeca and just in front of the Malpighia tubes, there are refractile oval inclusions which colour faintly with ninhydrin and sometimes wit fat stains. The nature of these is uncertain; they do not appear to consist solely of protein.

As starvation proceeds the aqueous vacuoles in the fat body become smaller and ker and probably increase in number (Fig. 3). Fig. 4 B shows two living cells in rva starved 7 days. The contents of most of the vacuoles are homogeneous, but ome there are minute refractile particles, elongated crystals or clusters of needles n Brownian movement. On fixation in aqueous Bouin masses of such crystals, ying with them more or less grey or amber pigment, separate out in the form of res of radiating needles or double spheres or 'wheatsheaf' bundles of crystals . 4 B′). In the late stages of starvation, solid amber coloured deposits in the n of irregular spheres may separate during life. These crystalline deposits almost ainly consist of uric acid (see footnote, p. 60).

Thus during starvation, as the proteins are used up, the vacuoles of the fat body tain an increasing quantity of uric acid presumably in colloidal solution.

In the final stages of starvation the cells of the fat body may separate from one her and become rounded, many of the aqueous vacuoles shrinking or dis-earing.

Formation of reserves after feeding with carbohydrates

Starch. Within 6 hr. of feeding these fully starved larvae on starch, deposits of *ogen* appear in the cells of the posterior half of the midgut and there may be es of glycogen in the cells of the fat body in the front of the prothorax.

At 12 hr. there are granules of glycogen in some of the cells of the caeca and in ring of midgut cells immediately behind these. Then follows the region of clear ; in these glycogen is absent or present only as a few sparse granules. Halfway g the midgut heavy deposits begin abruptly; they are very plentiful throughout pyloric half. Glycogen is increasing in the prothorax but there is little elsewhere he fat body. Scattered granules are beginning to appear in the sarcoplasm of e muscles and in the ganglia of the nerve cord.

Subsequently (24 hr. to 6 days) glycogen increases progressively in amount but ins the same distribution. That is, in the gut it is almost confined to the caeca the cells just behind them, and to the pyloric half of the midgut where great sses of glycogen accumulate in the course of 2 or 3 days (Fig. 5 A). It occurs oughout the fat body and after several days it fills the greater part of each fat y cell (Fig. 6 A–D). The deposits in the muscles become gradually more dense by the 3rd or 4th day the sarcoplasm is almost solid with glycogen (Fig. 6 E–H). cogen occurs throughout the central nervous system, both in the ganglia and he connectives. But it is absent from the oenocytes, pericardial cells, epidermis imaginal disks.

In some larvae glycogen is present in the form of fine granules in the cells of the lpighian tubes, particularly in the proximal region; though sometimes there are ensive deposits throughout the tubes (Fig. 5 C). These deposits are not constant; they may appear within 12 hr. of feeding, at a time when glycogen is still absent n the abdominal fat body. It seems therefore almost certain that they are ived from the gut contents which presumably may on occasion enter the Mal-hian tubes and be there absorbed. Occasionally small deposits appear in the s of the hindgut, suggesting some absorption here.

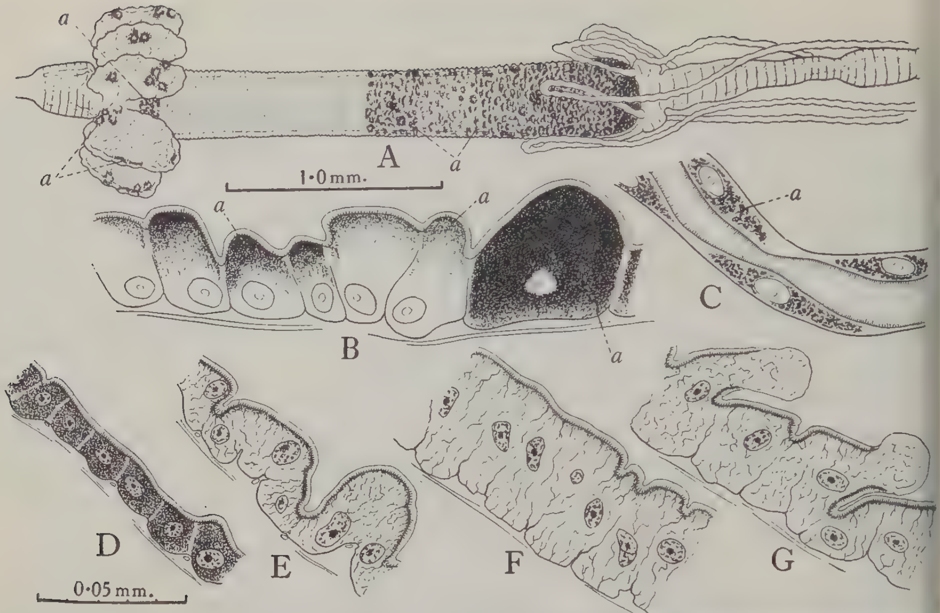


Fig. 5. A, gut of larva fed 2 days on starch; B, optical section of pyloric segment of midgut in th larva showing distribution of glycogen in the cells; C, portion of Malpighian tube of larva fe 24 hr. on sucrose showing glycogen in the cells; D–G, longitudinal sections of pyloric half of midgu (fixed in Carnoy, stained haematoxylin and eosin); D, in starved larva; E, larva fed 12 hr. on starch F, larva fed 3 days on starch; G, the same showing droplets filled with glycogen protruding throug the striated border. *a*, glycogen stained with iodine.

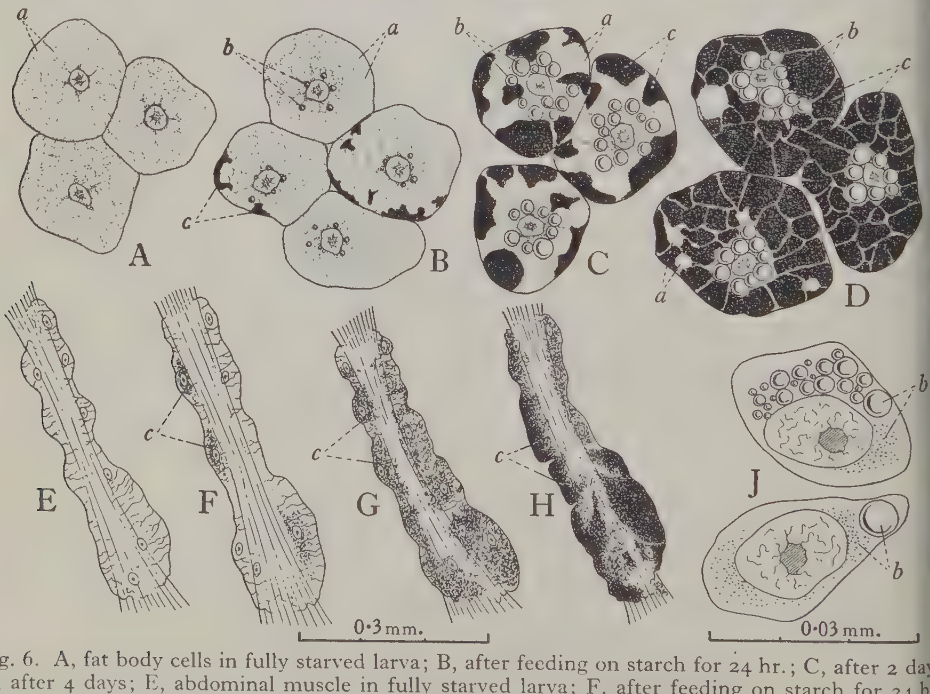
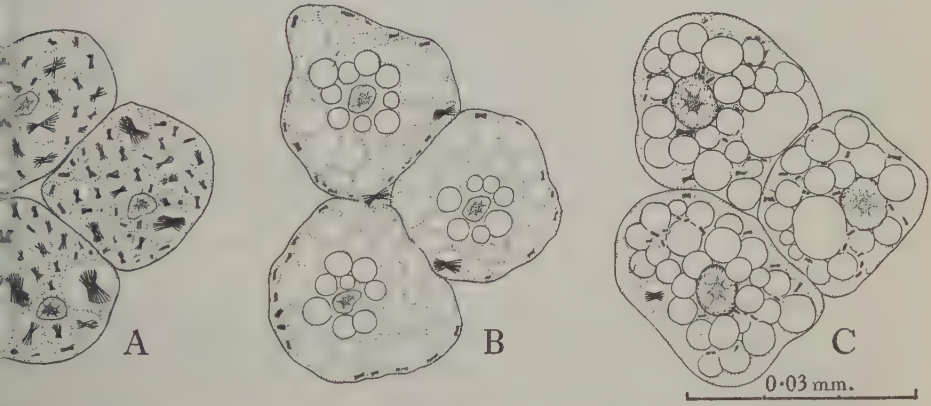


Fig. 6. A, fat body cells in fully starved larva; B, after feeding on starch for 24 hr.; C, after 2 day D, after 4 days; E, abdominal muscle in fully starved larva; F, after feeding on starch for 24 hr. G, after 2 days; H, after 4 days; J, oenocytes after feeding on starch for 2 days. *a*, watery vacuoles *b*, fat droplets; *c*, glycogen stained with iodine.

s to the location of the glycogen in the cells; in the pyloric half of the midgut
gen occurs in diffuse form in the cytoplasm. It first appears in a layer im-
ately below the striated border (Fig. 5 B); but later, and in many of the cells,
y extend and accumulate until the swollen cell is practically solid with glycogen
5 B). Sections of such cells show a delicate meshwork of cytoplasm with the
stices filled with glycogen (Fig. 5 F). Very often there are large droplets of
lasm filled with glycogen protruding through the striated border (Fig. 5 G).
probable, however, that these are artefacts of fixation: I have been unable to
hem in optical sections of the living larva.[1]

imilarly, in the cells of the fat body, glycogen is first seen in vacuoles (and
ps in the cytoplasm) at the periphery of the cells (Fig. 6 B). Later these
les, with delicate strands of cytoplasm lying between them, almost fill the
cell (Fig. 6 D). Meanwhile the uric acid vacuoles are displaced, chiefly to
uter surface (Fig. 7 B), and the total quantity of uric acid present appears to
eatly reduced.



A, uric acid crystals in fat body cells of fully starved larva (fixed in aqueous Bouin); B, in a similar larva fed for 3 days on starch; C, in a similar larva fed for 3 days on casein.

fter feeding on starch, *fat* first makes its appearance in the oenocytes. It is
here between 24 and 48 hr. from the start as a diffuse staining in which no
cles can be resolved, as a fine 'dust' or as small scattered droplets (Fig. 6 J).
out the same time fat droplets appear in the fat body at the front of the pro-
x and later in the fat body elsewhere. They may occur in cells from which
gen is still absent. They arise first as minute droplets close to the nuclear
brane (Fig. 6 B) and even in the later stages when these fat droplets are en-
l and the cell is filled with glycogen they generally retain this distribution
6 D). Occasional droplets of fat occur in the cells of the caeca and the anterior
f the midgut.

here is of course no accumulation of *protein* during starch feeding. The
lasm remains very deficient in protein and the nuclei remain small.

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roplets of this type were described by van Gehuchten (1890) in *Ptychoptera* as secretory
s. They are regarded by Vignon (1899) in *Chironomus*, by Steudel (1913) in various insects,
de Boissezon (1930) in *Culex* as artefacts.

Sugars. The question arises whether the deposition of glycogen in the cells of the pyloric half of the midgut is a specific function of the cells in this region, or whether it is merely the result of digestion of starch being delayed until it reaches this segment of the gut.

That there is a real difference in the absorptive or storage function of the cells is suggested by the fact that some glycogen is deposited also in the cells of the caeca and in the ring of cells just behind them. A further proof has been obtained by feeding the larvae on glucose in 2·5 % solution. The deposition of glycogen in the gut has exactly the same distribution as after feeding with starch: after 6 hr. feeding there is a sharp boundary between the pyloric half of the midgut which contains plenty of glycogen and the anterior half without.

This rapid deposition of glycogen in the gut wall after feeding with carbohydrates provides a means of comparing the ability of the mosquito larva to deal with different sugars and sugar alcohols. These have been given in 2·5 % solution to batches of twelve larvae which have been fixed and dissected after 6 and 12 hr.

The results prove clearly enough that even among sugars which can serve as a source of glycogen some are more efficient than others; they seem to form a graded series in this respect. There are other sugars which do not yield any glycogen at all. The individual variation is such that accurate grading is impossible; but the sugars tested have been roughly sorted into five groups.

(*a*) *Glucose* is definitely the most efficient. At the end of 6 hr., besides the deposits in the gut, there is plenty of glycogen in the fat body in the prothorax and elsewhere and it is beginning to accumulate in the sarcoplasm of the muscles. There are massive accumulations at the end of 12 hr.

(*b*) *Fructose* does not lead to quite such a rapid deposition as glucose, but plenty of glycogen appears in the prothoracic fat body within 12 hr. The disaccharides *sucrose* and *maltose* fall into this group, being only slightly less efficient than glucose.

(*c*) *Galactose* produces fairly large deposits in the pyloric half of the midgut within 12 hr., but no glycogen has appeared in the fat body. Other substances falling into this group are: *mannose*, *trehalose*, *α-methyl-d-glucoside*, *mannitol*, *sorbitol* and the disaccharide *lactose*.

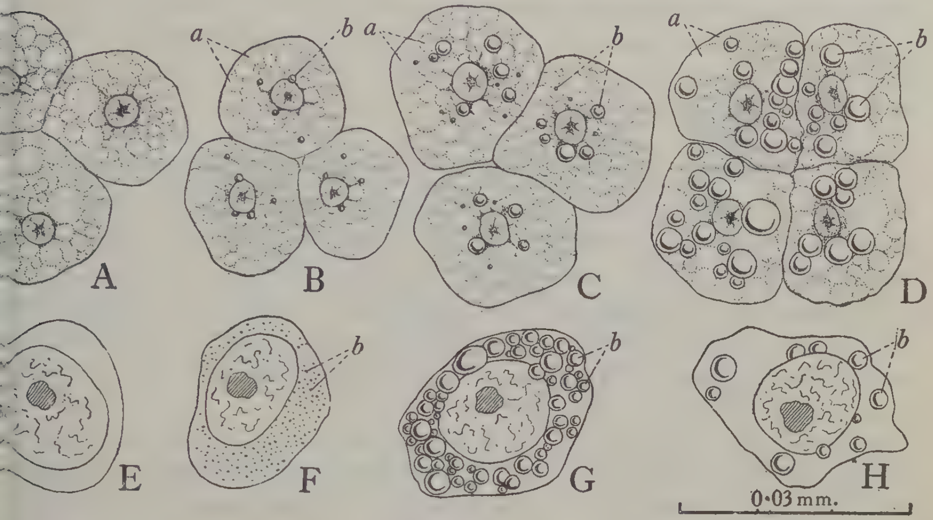
(*d*) Sugars which lead to the formation of only very small traces of glycogen in the gut in 12 hr. are the pentose *xylose*, the sugar alcohol *dulcitol* and the disaccharide *cellobiose*.

(*e*) No glycogen is formed from the hexose *sorbose*, the pentoses *arabinose* and *rhamnose* or the trisaccharide *raffinose*.

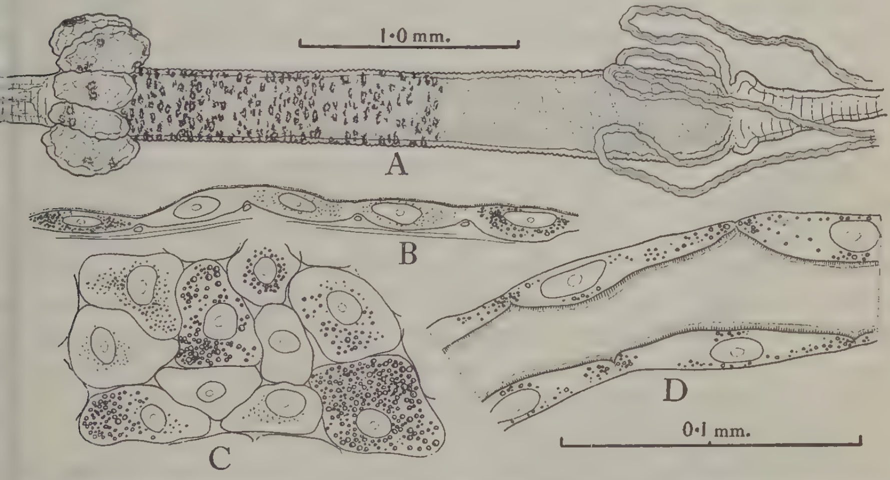
Formation of reserves after feeding with fats

Within 24 hr. of feeding with olive oil minute droplets of fat are usually present throughout the fat body, lying chiefly, though not exclusively, close to the nuclear membrane (Fig. 8 B). Exceedingly fine particles of fat are present also in the oenocytes and may appear in these cells at a time when none is laid down in the fat body (Fig. 8 F). In later days there is a progressive increase in the fat droplets in these tissues.

the gut, fat is confined to scattered cells in the caeca and to the clear cells of nterior half of the midgut (Fig. 9 A). 24 hr. after feeding with olive oil the of this region contain abundant fat: some are filled with fairly large droplets,



A, fat body cells in fully starved larva; B, after feeding on olive oil for 24 hr.; C, after 2 days; r 3 days; E, oenocyte in fully starved larva; F, after feeding on olive oil for 24 hr.; G, after (in this larva the oenocytes contained an exceptionally large amount of fat); H, after 3 days. ry vacuoles; *b*, fat droplets.



A, gut of larva after feeding on olive oil for 24 hr., showing fat in caecal cells and anterior midgut; B, optical section of the midgut epithelium showing fat in the cells; C, the same in view; D, portion of Malpighian tube showing fat droplets in the cells.

show only a fine 'dust' and yet other cells contain no fat at all (Fig. 9 B, C). ne larvae, on the other hand, there may be fat in the cells of the fat body but deposited in stainable form in the gut wall. In a few larvae minute fat droplets r in the cells at the lower ends of the Malpighian tubes (Fig. 9 D).

At no stage does there seem to be any accumulation of glycogen in the tissue after feeding with olive oil.

Similar results are obtained after feeding with egg 'lecithin', but absorptio perhaps occurs more readily. In some larvae droplets of fat, besides filling th anterior half of the midgut, were plentiful in the caecal cells and in the cells of th cardiac region which secrete the peritrophic membrane. A barely resolvable fa 'dust' could sometimes be seen in the blood within the heart.

Formation of reserves after feeding with protein and amino acids

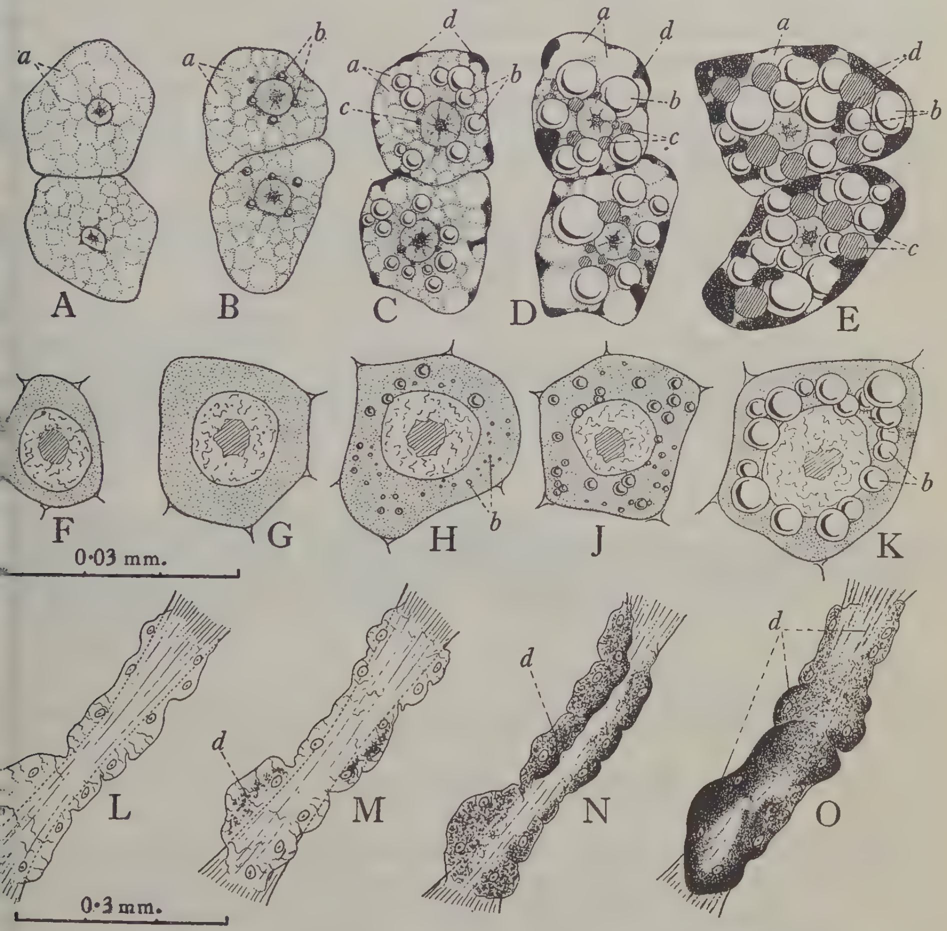
Casein. In larvae starved until all fat and glycogen have been used up, th proteins in all the tissues are largely wasted. Everywhere the nuclei are very small the nucleoli deficient in protein. In the fat body there is a little protein in the form of strands radiating from the nucleus, but elsewhere the protein meshwork between the vacuoles in these cells is almost invisible (Fig. 10 A). The oenocytes are similarly wasted (Fig. 10 F), protein in the muscles is reduced and the epithelium of the midgut is greatly attenuated (Fig. 11 A, A′).

Within 24 hr. of feeding on casein the nuclei in the fat body are enlarged, the protein meshwork, particularly around the nucleus, is more evident, and everywhere the tint given with ninhydrin is much darker. Minute droplets of fat may have appeared, chiefly in the neighbourhood of the nuclear membrane, but little glycogen is present as a rule except in the prothorax (Fig. 10 B). The oenocytes may show a very fine fat 'dust' or scattered droplets or they may still be free from fat (Fig. 10 G). The sarcoplasm of the muscles may show a little glycogen finely dispersed (Fig. 10 M) and traces appear in the central nervous system. The cells of the gut epithelium are becoming enlarged but they contain neither fat nor glycogen (Fig. 11 B, B′).

After 2 days, protein in the cytoplasm of the fat body cells is further increased and in the thorax minute spheres of protein are making their appearance on the nuclear membrane. There are filaments in the nucleus radiating from the plasmosome to the nuclear membrane and in many places the protein droplets have the appearance of being attached to these filaments. Fat droplets are larger and more plentiful and are often scattered throughout the cells (Fig. 10 C). Glycogen occurs in many of the fat cells, chiefly at the periphery, but not in all; it is most abundant in the prothorax. The oenocytes are further enlarged and contain small fat droplets (Fig. 10 H). Glycogen is increasing in the sarcoplasm of the muscles (Fig. 10 N) and in the nervous system, and traces may occur throughout the midgut.

After 3 days the droplets of protein in the fat body cells are enlarged and plentiful everywhere. Although many of them still rest upon the nuclear membrane, others are dispersed throughout the cell among the enlarged fat droplets (Fig. 10 D). Glycogen is plentiful throughout the fat body; it occurs chiefly in the outer parts of the cell in deposits distorted by other cellular inclusions and sometimes in vacuoles with sharply defined boundaries. Fat droplets in the oenocytes (Fig. 10 J) and glycogen in the sarcoplasm and in the other tissues mentioned continue to increase. Glycogen may occur also in the cells of the Malpighian tubes.

From sections stained with haematoxylin and eosin there seems little doubt that protein spheres in the fat body arise from the nucleus. They appear first as ute basophil droplets on the nuclear membrane and are often seemingly con- ted by radiating filaments with the nuclear plasmosome. As they enlarge and s outwards into the cytoplasm they become eosinophil.



10. A, fat body cells in fully starved larva; B, the same in larva after feeding on casein for r.; C, after 2 days; D, after 3 days; E, after 7 days; F, oenocyte in fully starved larva; G, after ng on casein for 24 hr.; H, after 2 days; J, after 3 days; K, after 7 days; L, abdominal muscle lly starved larva; M, after feeding on casein for 24 hr.; N, after 2 days; O, after 5 days. *a*, watery oles; *b*, fat droplets; *c*, protein droplets; *d*, glycogen stained with iodine.

After 5 or 6 days the cells of the fat body are almost filled with refractile spherical usions (Fig. 10 E). In the living cell these cannot be differentiated into fat and tein and both sorts blacken with osmic acid. But when stained with fat stains with ninhydrin there is a sharp division into protein and fat droplets. Between e are a few distorted aqueous vacuoles; and also interspersed among them, but chiefly around the periphery of the cells are numerous deposits and ill-defined vacuoles of glycogen. It is probable that glycogen occurs also in the cytoplasmic framework of the cell. The oenocytes contain plenty of fat, embedded in the dense protein rich cytoplasm, but no glycogen (Fig. 10 K). The sarcoplasm is in many places practically solid with glycogen and there are elongated deposits between the muscle fibrils (Fig. 10 O). The midgut epithelium is much thickened and, particularly in the posterior half, very rich in protein (Fig. 11 C, C′). All parts of the gut wall may contain small amounts of glycogen, but the largest deposits occur at the hind end. There is some glycogen in the epidermal cells and in the cells of the oesophageal invagination. The pericardial cells have by now increased to nearly three times their diameter in the starved larva; they contain much protein but no fat or glycogen.

Larvae fed for several days with casein and then fixed in aqueous Bouin have the uric acid vacuoles dispersed among the various other inclusions (Fig. 7 C). The uric acid is probably reduced in quantity, but this has not been established with certainty.

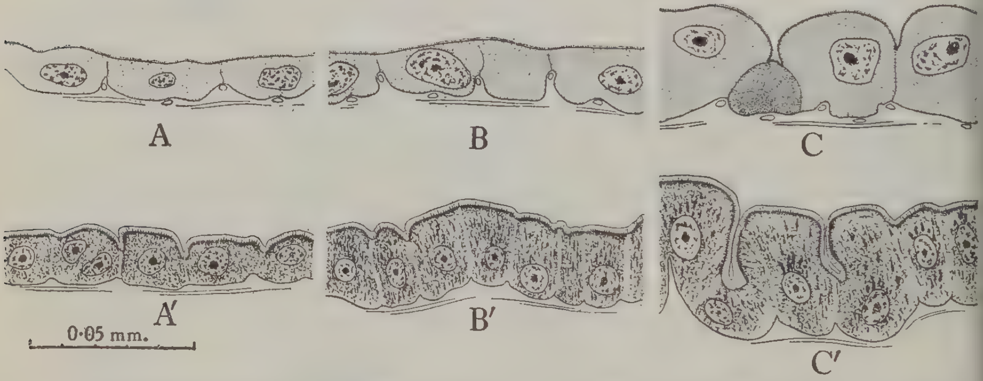


Fig. 11. Longitudinal sections of midgut of larvae; A, B, C, anterior segment; A′, B′, C′, posterior segment. A, A′, in fully starved larva; B, B′, after feeding on casein for 24 hr.; C, C′, after 3 days.

Amino acids. Of the readily available amino acids cystine and tyrosin are so insoluble that they are probably not absorbed rapidly enough to supply the energy requirements of the larva. For when starved larvae are fed on these substances they die, apparently of starvation, in about 48 hr. Glycine, on the other hand, while freely soluble, has such a small molecular weight that solutions of adequate strength are hypertonic and cause shrinkage of the larva. Alanine in 1·5% solution with kaolin and glutaminic acid 2·5% are satisfactory foods. The latter is more than saturated so that there is plenty of solid amino acid present. Both mixtures are adjusted to *p*H 4·0, the alanine with phosphoric acid, the glutaminic acid with sodium bicarbonate, and they are made up fresh every 12 hr.

After feeding on alanine for 48 or 60 hr. there may be minute quantities of visible fat in the fat body of a few larvae but most show no fat. Glycogen on the other hand is plentiful. It is present within 24 hr. in some of the cells at the pyloric of the midgut and there are traces in the ganglia. By 60 hr. there is abundant ogen in the pyloric half of the gut, it is present in some cells of the gastric caeca occasionally in traces in the Malpighian tubes. It is plentiful also in the ganglia connectives of the central nervous system, in the sarcoplasm of the muscles and ughout the fat body.

Glutaminic acid gives identical results, though perhaps rather more larvae elop visible fat in the fat body cells, and glycogen in the gut epithelium and in fat body may not be so conspicuous.

Storage of uric acid in the fat body

Under the conditions in which larvae have been reared in these experiments, re they have developed without interruption in a rich mixture of foods, there s we have seen, very little uric acid in the fat body at the time of moulting to fourth stage (Fig. 4 A, A′). After fasting for 24 hr. there is a great increase, and continues throughout starvation (Figs. 4 B′, 7 A).

This accumulation of uric acid has been regarded as evidence that the con- ption of protein for energy purposes takes place from the outset during star- on (p. 62). This belief is supported by the fact that the accumulation of uric is greatly reduced and sometimes seems to be entirely prevented if the larva is continuously from the time of moulting. And this is so whether the larvae are on the mixed infusion, on casein alone or on starch alone.

It is obvious from preparations such as that shown in Figs. 4 B′ and 7 A that total amount of uric acid collecting within the fat body cell during starvation reater than can have been produced from the protein originally present in the . Uric acid or precursors of uric acid must be taken up from the blood. When ae are fed with solid uric acid during starvation, the quantity of uric acid in the body cells is not obviously greater than that accumulating during simple vation; and if newly moulted larvae are fed on a mixture of casein and uric acid, uric acid appearing in the fat body is much less than that in unfed larvae.

Although this evidence cannot be regarded as conclusive, it thus seems likely the uric acid which collects in the vacuoles of the fat body is the product of mination within the cell of amino acids or proteins brought from elsewhere.

During starvation, when the reserve substances are diminishing, conditions in cells of the fat body are presumably ideal for the accumulation and persistence of acid within the vacuoles. When the starved larva is fed on starch or protein quantity of uric acid is reduced (Fig. 7). Presumably the physico-chemical or hanical state of the cell now favours the discharge of uric acid into the blood its consequent elimination by the Malpighian tubes.

DISCUSSION

Storage in different tissues

The results described in this paper emphasize the important part played by er tissues besides the fat body in storing reserves. The muscles, the oenocytes, gastric caeca and the posterior half of the midgut are particularly important as reserves of protein. The masses of glycogen laid down in the sarcoplasm of the muscles are at least as great as those in the fat body; while smaller amounts are stored in the nervous system, epidermis, gut and Malpighian tubes.

De Boissezon (1932) has stated that in larvae of *Culex* glycogen is confined to the fat body; but the widespread distribution observed in *Aedes* agrees with that described by Bogojawlensky (1935) and Paillot (1938) in the silkworm. In the silkworm the amount of glycogen in the muscles is quite small; a larger amount occurs in the muscles in *Ephestia* (Zeller, 1938); but in no insect have massive accumulations, such as are seen in *Aedes*, been recorded.

In the oenocytes of *Aedes* glycogen is consistently absent; but it has been recorded as occasionally present in the oenocytes of *Bombyx* (Bogojawlensky, 1935); while it is plentiful in those of aquatic Hemiptera (Poisson, 1924), in *Polistes* and *Melasoma* (Pardi, 1939*b*) and in several other insects (Hollande, 1914*a*).

As in all insects, fat is stored in *Aedes* chiefly in the fat body. Some is laid down in the oenocytes—as has been noted also by Poisson (1924) in aquatic Hemiptera. And when the food contains much fat this may collect in large quantities in the epithelium of the gastric caeca and the cardiac half of the midgut. Hobson (1931) observed a storage of fat in both anterior and posterior segments of the midgut of *Lucilia* larvae.

Influence of the nature of the food on the reserves

When the larva is fed on casein alone, the reserves in the fat body and elsewhere do not differ from those formed in larvae feeding on the normal diet of microorganisms: abundant protein, fat and glycogen are laid down. This confirms the evidence of other authors that protein in insects may serve as a source of both fat and glycogen. Thus Kulz (1881) showed that *Calliphora* larvae can form glycogen from egg albumen and Weinland (1907) supported this conclusion; while Pardi (1938 *a*, *b*) noted that at the end of larval life in *Polistes* and *Melasoma* glycogen first makes its appearance in the form of granules actually inside the protein globules. Weinland (1909) and Mishikata (1922) have shown that *Calliphora* larvae will lay down fat on a diet of fat-free fibrin or egg albumen.

Starch and other carbohydrates lead to very large accumulations of glycogen and, to a lesser extent, of fat. Fat, on the other hand, although it can obviously serve as a source of muscular energy, does not lead to the deposition of visible glycogen.

We have seen that the sugars differ much in the readiness with which they are transformed into glycogen. Comparative results of this kind have been obtained by Vogel (1931) on bees, Haslinger (1936) and Fraenkel (1940) on blowflies (*Calliphora*) and Hinman (1933) on newly hatched mosquito larvae (*Aedes*) by noting the time of survival of insects fed solely upon different sugars. The results for bees and flies have been brought together by Fraenkel. When compared with the present results on *Aedes* it may be noted that in all these insects glucose, fructose, sucrose and maltose are the most readily utilized sugars; in none is sorbose used; and for all of them the most available of the pentoses is xylose. Apart from this there are iking differences which need not be reviewed here. *Aedes* larvae, like *Calliphora* ults show no sign of the ‘toxic’ effect exerted by mannose on bees, an effect which ascribed by Staudenmayer (1939) to ‘competitive inhibition’ of enzyme action. r is there any sign of sorbitol having exceptional value as it seems to have for th bee and blowfly.

During starvation in *Aedes* protein, fat and glycogen are used up concurrently; c acid begins to collect in the fat body at once and larvae starved until all their has been consumed may still have a little glycogen in their muscles. Other ects show differences in these respects. In the cockroach glycogen is used first, n fat and finally protein; only during this final stage do the uric acid concretions the fat body increase (Philiptschenko, 1907). In *Dytiscus*, protein is used in large ounts from the outset (Pilewiczówna, 1926). Other examples are reviewed else- ere (Wigglesworth, 1939).

Fat body inclusions

(i) *Fat droplets.* These are the most obvious of the inclusions. They usually ear first around the nucleus, at least after feeding on starch or protein. Quite en when the fat first becomes visible it is in the form of a slender crescent applied a spherical non-fatty inclusion in this position. Large crescentic fat drops, which sometimes seen, are certainly artefacts occurring in badly fixed material; some ubt therefore exists about the genuineness of the small crescents. They have not en detected in living larvae.

It may be noted that Popoff (1910) (quoted by Schreiner, 1915) has stated that he fat body of muscids the fat droplets are derived from ‘chromidia’ discharged ough the nuclear membrane and Schreiner (1915) holds the view that fat plets in cells (in *Myxine*) are produced by mitochondria which are themselves duced by discharge from the nucleolus. The mitochondrial origin of fat droplets *Culex* is supported by de Boissezon (1930).

(ii) *Protein droplets* certainly arise in *Aedes* in contact with the nuclear mem- ne. Berlese (1901) and Pérez (1910) have described this in many insects. They en appear in *Aedes* as though connected to the nucleolus by filaments. But ir origin is a subject of much controversy. Schreiner (1916, 1918) describes ments connecting granules in the cytoplasm (which he regards as precursors of mitochondria) to the nucleolus; and Ludford (1925) has described the apparent charge of nucleolar substance into the cytoplasm in living cells in tissue culture. Boissezon (1930) and Hosselet (1931) in the fat body of *Culex* and Paillot & el (1926, 1928) in *Pieris* and *Bombyx* hold that the mitochondria produce the tein inclusions. Bishop (1922, 1923) describes the nuclear membrane in *Apis* breaking down and the basophil nucleoli as being set free into the cytoplasm to e rise to the protein droplets. But no such rupture could be seen by Schnelle 23) and Pardi (1935) has shown that the basophil cytoplasmic inclusions around nucleus do not stain by the Feulgen method. In reviewing the subject Pardi 39*a*) considers that it is not proved that the nucleus gives rise to either fat or tein inclusions.

The nature of the protein droplets has been equally disputed. Possibly this varies in different insects. Kreuscher (1922) describes the protein droplets in *Dytiscus* as replacing the fat droplets almost entirely before pupation; and Pérez (1920), Schnelle (1923) and Schmieder (1928) all take the view that the 'albuminoid granules' are formed largely by transference of material from the fat droplets. Zakolska (1928) refers to these granules in *Tenebrio* as 'albumino-fatty granules' because they give reactions for both fatty acids and proteins.[1] Bishop (1922, 1923) likewise believes that in *Apis* the fat contents of the cell are incorporated with the protein to form a common raw material for tissue use. His evidence is the blackening of the protein droplets with osmic acid. Pardi (1939*a*) finds some support for this belief in young larvae of *Melasoma* where the protein globules stain with Ciaccio's stain. But there is no sign of this in older larvae, nor in *Polistes*, *Pieris* or *Aporia*. Zeller (1938) describes the highly refractile protein inclusions in *Ephestia* as staining yellowish brown with osmic acid. In *Aedes* also, as we have seen, they darken in osmic acid, although they fail to stain with fat stains. Perhaps this merely indicates that a certain amount of lecithin is incorporated with the protein. There is certainly no obvious reduction in the fat droplets as the protein droplets enlarge.

(iii) *Glycogen* is much less sharply differentiated than the other inclusions. Sometimes it seems to be diffused in the cytoplasmic framework of the cell. But usually it is confined to vacuoles. These, however, appear to have a low interfacial tension and are so readily deformed by the fatty and protein droplets that their boundaries are not always easy to make out. As described by Bogojawlensky (1935) in the silkworm and by Zeller (1938) in *Ephestia* these irregular deposits occur chiefly at the periphery of the fat body lobes.

(iv) *Watery vacuoles* in the fat body have been described by Voinov (1927) in *Chironomus*. She likewise often observed a 'dense secretory granule' in Brownian movement in the vacuolar fluid. She states that neighbouring vacuoles may fuse until a single enormous vesicle fills the cell. Hosselet (1931) supposes that, in *Culex*, enzymes from the nucleus are concerned in the formation of these vacuoles which are described as later migrating to the periphery of the cell, fusing to larger vacuoles, becoming smoky in tint and finally 'approximating to urates' (cf. Hollande, 1914*b*).

In *Aedes*, as we have seen, uric acid collects in the vacuoles chiefly during starvation; and this was attributed to the endogenous production of uric acid from proteins in course of deamination. Hollande (1914*b*, 1925) came to the same conclusion when he found, as Philiptschenko (1907) had already found in the cockroach that injection of urates into the blood of Orthoptera or larvae of *Vanessa* does not lead to any increase in uric acid in the urate cells of the fat body. In *Polistes* uric concretions become very numerous in the urate cells at the time when glycogenic activity in the fat body at the expense of the reserve proteins is at its maximum (Pardi, 1939*a*). Even if the vacuoles have a nuclear origin there is no reason to suppose that the uric acid collecting in them is derived solely from purines in the nucleus as de Boissezon (1930) suggests.

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[1] Fatty acids were demonstrated with Nile blue and by Fischler's method, both of which are regarded by many writers as non-specific.

Importance of gut and Malpighian tubes in intermediary metabolism

Van Gehuchten (1890) regarded the clear cells of the anterior half of the midgut *Ptychoptera* as absorbing, the granular cells of the posterior half as secreting. Boissezon (1930) showed that absorption of iron saccharate is limited to the terior half of the midgut in *Culex* larvae. If the deposition of absorbed sub-nces can be taken as an index of the site of absorption, it would seem from the sent experiments that, in *Aedes*, fat, sugar and amino acids are absorbed in the ca, that the clear cells of the cardiac half of the midgut absorb fat, and the nular cells of the pyloric end sugar and amino acids, while all three may be orbed by the Malpighian tubes if the gut contents happen to enter them.

But more important is the light these results throw on the part played by the in intermediary metabolism. It is claimed by Bogojawlensky (1935) that on ding starved silkworm larvae, glycogen appears first in the fat body and later in other organs beginning with the midgut epithelium. He concludes that sugars absorbed from the gut and first synthesized to glycogen in the fat body. But *Aedes* larvae it is quite certain that sugars of many sorts are converted into cogen in the epithelium of the pyloric half of the midgut before any appears in fat body, and its deposition in the fat body may synchronize with its appearance he ganglia and muscles.

This same sequence is observed after feeding with alanine and glutaminic acid, ch can evidently be deaminated and converted into glycogen in the midgut helium. This recalls that Brown & Farber (1936) and Brown (1938) produced lence of a deaminase for peptones in the posterior part of the midgut of blowfly ae and this enzyme has recently been shown by Dr H. Hurst[1] to act also on free no acids. On the other hand, after feeding with casein, fat and glycogen are ally laid down in the fat body before they appear in the gut wall.

Glycogen formation both from the various sugars and from amino acids is sible also in the Malpighian tubes; though only in a few larvae apparently do the contents pass into them.

SUMMARY

In the newly moulted fourth stage larva of *Aedes aegypti* the cells of the fat y contain fat droplets and numerous watery vacuoles. Protein is present only he form of cytoplasmic strands between these inclusions. Some of the watery uoles contain glycogen; in others there is uric acid in solution. Glycogen is sent also in the ganglia and connectives of the central nervous system and in at masses in the sarcoplasm enveloping the muscles and between the muscle ils.

During starvation, fat, glycogen and protein are used up concurrently. In 15 days (at 28° C.) all the stainable fat has disappeared and glycogen is absent present in minute traces in the sarcoplasm only. Nuclei and cytoplasm in all tissues are much wasted. The utilization of protein is marked by a progressive accumulation of uric acid in the aqueous vacuoles of the fat body. In some of these uric acid may crystallize out during life. This accumulation of uric acid can be largely prevented by feeding on casein or on starch.

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[1] Unpublished work to which I am kindly permitted to refer.

When starved larvae are fed on starch there is a massive deposition of glycogen in the epithelium of the *posterior half* of the midgut. A little is deposited in the cells of the gastric caeca and occasionally in the cells of the Malpighian tubes. Subsequently glycogen collects in the cells of the fat body until these are enormously distended with it and around the muscles until the sarcoplasm is practically solid with glycogen. Some appears also in the central nervous system. Rather small fat droplets collect round the nuclei of the fat body and in the oenocytes.

After feeding on sugars, glycogen and fat have the same distribution as above. Different sugars vary in their efficiency as precursors of glycogen; from some (raffinose, sorbose, rhamnose, arabinose) no glycogen seems to be formed.

After feeding on olive oil, droplets of fat are limited to the cells of the *anterior half* of the midgut and to some cells in the gastric caeca. Occasionally droplets appear in the cells of the Malpighian tubes. Much fat collects in the oenocytes and in the fat body, but no glycogen is laid down.

After feeding on casein there is a rapid increase in protein in the cytoplasm of all the tissues and the nuclei enlarge. Minute droplets of fat appear in the fat body, chiefly around the nuclei, and later pass outwards and enlarge. Fat accumulates also in the oenocytes. Glycogen is deposited in large amounts in the cytoplasm and in indefinite vacuoles chiefly at the periphery of the fat body cells. It is laid down in great quantity in the sarcoplasm of the muscles, to a less extent in the central nervous system and later in the epidermis, Malpighian tubes and gut wall. Minute droplets rich in protein appear on the nuclear membrane of the fat body cells; they have the appearance of being discharged from the nuclei; gradually they enlarge and pass outward among the fat droplets. These protein droplets blacken with osmic acid but they do not stain with fat stains. They are basophil when first formed, becoming acidophil as they enlarge.

After feeding on alanine or glutaminic acid glycogen is laid down, as after carbohydrates, in the caecal cells, the pyloric half of the midgut, the central nervous system, sarcoplasm and fat body. Small amounts of fat become visible in the fat body.

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STUDIES ON NITROGEN FIXATION BY BLUE-GREEN ALGAE

I. NITROGEN FIXATION BY *ANABAENA CYLINDRICA* LEMM.

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(With Two Text-figures)

Recent work, particularly that of De (1939), has shown that some blue-green alga are capable of utilizing the free nitrogen of the atmosphere in the synthesis of cell material. Much further work will be necessary, however, before the importance o nitrogen fixation by these organisms in nature can be estimated, our knowledg both of the distribution of the property within the group of the Myxophyceae an of the physiology of the process being very vague.

The aim of the present paper is to present proof of nitrogen fixation by a specie of *Anabaena* which has not hitherto been examined from this point of view. Late it is hoped to publish the results of investigations on some physiological aspect of the fixation process in the same species.

1. ISOLATION OF THE ALGA

(*a*) *Source and identity of material*

The alga upon which this work has been carried out was isolated by Mr S. P. Chu of the Botany Department, Queen Mary College, London, from a small garden pond in Surrey. De's solution (De, 1939) was found to be a suitable culture medium.

This alga has been identified as *Anabaena cylindrica* Lemm. (Geitler, 1932). It differs from the original description of this species in that the curious mucilaginous envelopes, described by Lemmermann, surrounding the heterocysts have not been observed.

(*b*) *Isolation of the alga in pure culture*

Isolation in pure culture is a necessary preliminary to the study of nitrogen fixation in a given organism. Since it is possible that different physiological races of a species of alga may exist, it is desirable that the cultures used should have originated from a single individual. Such unialgal cultures of *Anabaena cylindrica* were obtained by isolating an individual filament with a sterile capillary pipette under the low power of the microscope and inoculating it into sterile culture medium. Cultures obtained by this method were, however, still contaminated with bacteria.

The difficulty of obtaining cultures of Myxophyceae free from bacteria has been stressed elsewhere (De, 1939). Various methods have been tried in the course of the present investigation, including repeated subculture on silica gel and irradiation with ultra-violet light. Bacteria-free cultures were eventually obtained by treatment of the alga with dilute chlorine water. A small portion of algal material was mmersed in chlorine water of a concentration of 25 p.p.m. for 2 min., washed in sterile water and plated out. Exhaustive examination failed to show the presence of contaminating organisms in this culture or in subcultures derived from it. All the cultures used in the subsequent work were grown from this culture. It has not so far been found possible to obtain bacteria-free cultures of any other species of blue-green alga by this method. Its success in this particular instance is thought to be due to the absence of spore-forming bacteria in the original material.

(*c*) *Tests of purity of the cultures*

The most reliable method of demonstrating the absence of bacteria in a given culture is by means of special culture media. De (1938) has pointed out two possible sources of error that must be borne in mind when using this method:

(1) The culture may include bacteria incapable of growing in the media used.

(2) Some bacteria may remain embedded in the mucilage of the alga, and, failing to pass out into the medium, will not produce visible contamination.

In order to minimize the first source of error a range of media suitable for a wide variety of physiological types of bacteria was used. The standard method of reparing the inoculum for a bacteriological examination of an algal culture was to shake a sample of the material vigorously for 5 min. with sterile glass beads and about 10 ml. of De's medium in a sterile tube closed with a sterile rubber stopper. Drops of this suspension were then used to inoculate the test cultures. Tests have been made with the following media:

(1) Caseinate agar (Taylor, 1940).

(2) Lemco-peptone agar.[1]

(3) Sucrose-nitrate-soil-extract agar (De, 1939).

(4) Albuminate agar (Waksman, 1922).

(5) Sucrose-nitrate-soil-extract liquid medium (De, 1939).

(6) Litmus milk.

(7) Dextrose peptone broth (1 % each of dextrose and "Difco" proteose peptone).

(8) Bacto-tryptone broth ('Difco', 1 % solution).

(9) 2 % mannite in tap water + 0·02 % potassium monohydrogen phosphate a medium specially suited for *Azotobacter*).

Incubation was in the dark at a temperature of 25 or 30° C. for a period of 3 weeks. n the case of bacto-tryptone broth both aerobic and anaerobic cultures were set up. Test cultures were always prepared in triplicate.

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[1] The composition of this medium, for which I am indebted to Miss J. Meiklejohn, Rothamsted, as follows: agar, 15 g.; 'Lemco' meat extract, 3 g.; peptone (bacteriological, B.D.H.), 10 g.; sodium chloride, 5 g.; distilled water, 1 l. After neutralization to phenolphthalein it is sterilized at atm. for 15 min.

In case the second source of error was operative, another method of preparin the inoculum was also used. A test-tube, 7·5 × 2·5 cm., containing some kieselguh and about 10 ml. of water was sterilized. Some algal material was introduced int this and ground up by means of a second sterile test-tube, 15 × 1·75 cm., used as pestle. Sterile cotton-wool was bound round the middle of the second tube i order to close the mouth of the first tube and so reduce risk of contaminatio during the process. It was sometimes necessary to subculture cultures prepare with this inoculum since the turbidity produced by the kieselguhr rendered the recognition of contamination impossible.

Bacterial contamination as indicated by turbidity in the liquid cultures o growth of colonies on solid media was absent in all cases. The media used showed themselves when tested to be perfectly capable of supporting bacterial growth. Direct microscopical examination of smears of algal material stained with carbol-thionin blue also supported the view that the cultures were bacteria-free.

2. METHODS

(a) *Culture methods*

(1) *The medium.* Growth of the purified alga in De's modification of Benecke's solution (De, 1939) being unsatisfactory, experiments were made to find a modification giving better results. It was found that vigorous growth could be obtained by adding ferric chloride in greater amount, the optimum being about 0·4 p.p.m. of iron. Since molybdenum has been found to be necessary for nitrogen fixation by *Azotobacter* (Burk, 1934) a trace of this element was added, though no significant improvement of growth was observed as a result. The composition of this modified medium is as follows:

Potassium monohydrogen orthophosphate ...	0·2 g.
Magnesium sulphate heptahydrate	0·2 g.
Calcium chloride decahydrate	0·2 g.
Ferric chloride anhydrous	1·2 mg.
Potassium molybdate (K_2MoO_4)	0·25 mg.
'Pyrex' redistilled water	1 l.

The chemicals used were of analytical quality. The medium was sterilized at 1 atm. for 15 min. in the autoclave and allowed to stand at least 48 hr. before inoculation. Its *p*H was 7·3.

(2) *Culture vessels.* 'Pyrex' conical flasks of 250 ml. capacity, plugged with cotton-wool, were used throughout this work. The flasks were cleaned with chromic acid, steamed and rinsed with 'Pyrex' distilled water before use.

(3) *Culture chamber.* The culture chamber used was the larger of the two described by De (1939). Owing to the heating effect of the lamps (which was reduced as far as possible by the interpolation of two sheets of ¼ in. plate glass between the lamps and the cultures), it was found impossible to maintain a constant temperature of below 30° C. by means of a Hearson capsule. Since a constant

mperature was not essential for this portion of the work, no attempt was made to gulate the temperature except that it was not allowed to drop below 21° C. The mperature remained at 21° C. for most of the time, rising to about 27° C. during llumination. Illumination was for a period of about 8 hr. daily.

(4) *Stock cultures.* These were maintained on De soil extract agar slopes in st-tubes. Before using a culture for preparing inoculum for a series of experiments was thoroughly examined for contaminants by inoculating, from a suspension repared by shaking with glass beads, into media, 1, 2, 5, 7 and 8 listed on p. 79.

(5) *Inoculation.* A portion of material from a young stock culture was shaken ith sterile medium in a sterile flask closed with a tightly fitting sterile rubber opper for 15 min. The heavier material was allowed to settle, and after decantation ortions of 0·25 ml. of the suspension were used as inoculum. The suspension repared in this manner was found to be sufficiently uniform for all ordinary rposes.

(*b*) *Chemical methods*

When the contents of a culture flask were to be analysed, the alga was first tached from the sides of the flask by means of a rubber-tipped glass rod, then the ga and medium were separated by centrifuging (at 2500 r.p.m.). The alga was ashed once with distilled water and the washings added to the medium. The edium was concentrated for analysis by evaporation in vacuo at *c.* 75° C. in Pyrex stilling flasks.

Total nitrogen was estimated by the micro-Kjeldahl method described by egl (1930). Nitrate and nitrite nitrogen was estimated by the micro-Kjeldahl ethod after reduction to ammonia by reduced iron in acid solution, a parallel ank determination, in which the iron was omitted, being made to allow for monia already present or produced by hydrolysis. Where nitrite or nitrate were esent total nitrogen was also determined after reduction. It was thought that, uring reduction in this manner, nitrites might perhaps react with amino acids esent to produce gaseous nitrogen and hence cause an error in the estimation, ut experiments showed that the method was perfectly reliable when nitrites and ino acids were present together. At all stages of the analyses and preparation for alysis, blank determinations were carried out and the results, which were usually all, subtracted from the results of the actual estimations.

3. THE NITROGEN-FIXING CAPACITY OF THE ALGA

Any experiment designed to show nitrogen fixation by a given organism must carried out with the following two points in mind:

(1) The organism must be in absolutely pure culture. Even if a contaminant present which is known to be otherwise incapable of fixing nitrogen, the possibility nnot be precluded that it may fix nitrogen in the presence of the organism which being examined.

(2) It must be absolutely certain that any increase in fixed nitrogen which takes ace in a culture is due to the uptake of free nitrogen. Ideally, a manometric

method, showing decrease in free nitrogen accompanying increase in fixed nitrogen should be used, but this is frequently inconvenient. A method showing increase in combined nitrogen is satisfactory provided that precautions are taken to exclude the possibilities that combined nitrogen may be supplied from an unsuspected source, e.g. the atmosphere, or that the apparent increase may be due to some defect in the method of estimation.

Since pure bacteria-free material of *Anabaena cylindrica* shows vigorous growth in a medium free from combined nitrogen, it is probable that it is able to fix nitrogen. It is possible, however, that nitrogen is being absorbed in the form of ammonia or oxides of nitrogen from the atmosphere. In order to show that this was not so in the case of the algae studied by him, De (1938) carried out an experiment in which air was bubbled through sulphuric acid to remove ammonia, and potassium bicarbonate to remove oxides of nitrogen, before passing over the cultures. In the course of the present work it has been found that gas washing bottles of the type used by De are quite inefficient for this purpose, and, in the absence of proof to the contrary, it must be assumed that all the combined nitrogen present in the air which passed over the cultures was not removed. De's contention (1939) that the increase in combined nitrogen observed in his cultures could not be due to absorption of ammonia or oxides of nitrogen from the atmosphere since uninoculated control flasks, exposed side by side with the cultures, did not show any increase of nitrogen, cannot be regarded as valid since a growing alga would form a very much more efficient absorbing system for such substances than the medium alone. It is unlikely that the comparatively large increases in combined nitrogen found by De in his cultures were actually due to absorption of fixed nitrogen from the atmosphere, but this defect in his methods introduces an element of uncertainty which is undesirable in an investigation of this kind.

An experiment similar to that of De, but using improved apparatus, has therefore been carried out with *Anabaena cylindrica*. Before passing over the cultures, air was purified from traces of combined nitrogen by passage through 1 % sodium bicarbonate solution (Fig. 1 *B*) and 25 % sulphuric acid (*D*), the wash bottles being of a type fitted with fritted glass bubblers. It was necessary to have the reagent with the lower vapour pressure (the sulphuric acid) nearer to the culture vessels since otherwise the cotton wool filter *E* became wet and allowed the cultures to become contaminated. The four culture flasks (*F*, *G*, etc.), one of which was left uninoculated as a control, were connected in series in the culture chamber. This part of the apparatus was sterilized and assembled under aseptic conditions and the air passing through it was sterilized by the sterile cotton wool filter *E*. A second sterile filter (*H*) and wash bottle containing sulphuric acid (*J*) were included respectively to prevent contamination and to absorb ammonia in the event of any sucking back. *A*, *C* and *I* were safety flasks. All connexions were sealed with paraffin wax. Air was drawn slowly (approximately 3 l. per hour) and continuously through the apparatus by means of a filter pump.

The efficiency of the gas-washing system was tested as follows. In each of two culture flasks was placed 100 ml. of *N*/50 sulphuric acid (to absorb ammonia) and each of two others 100 ml. of $N/50$ sodium hydroxide (to absorb oxides of itrogen). Air was drawn through the wash bottles and then through these flasks r a period of 10 days, the rate of flow being somewhat more rapid than that nployed for aerating cultures. At the end of the experimental period the contents the flasks were found to show no increase in combined nitrogen compared with ntrols analysed at the beginning of the experiment. This demonstrates that nmonia and oxides of nitrogen were effectively removed from the air passing over e cultures.

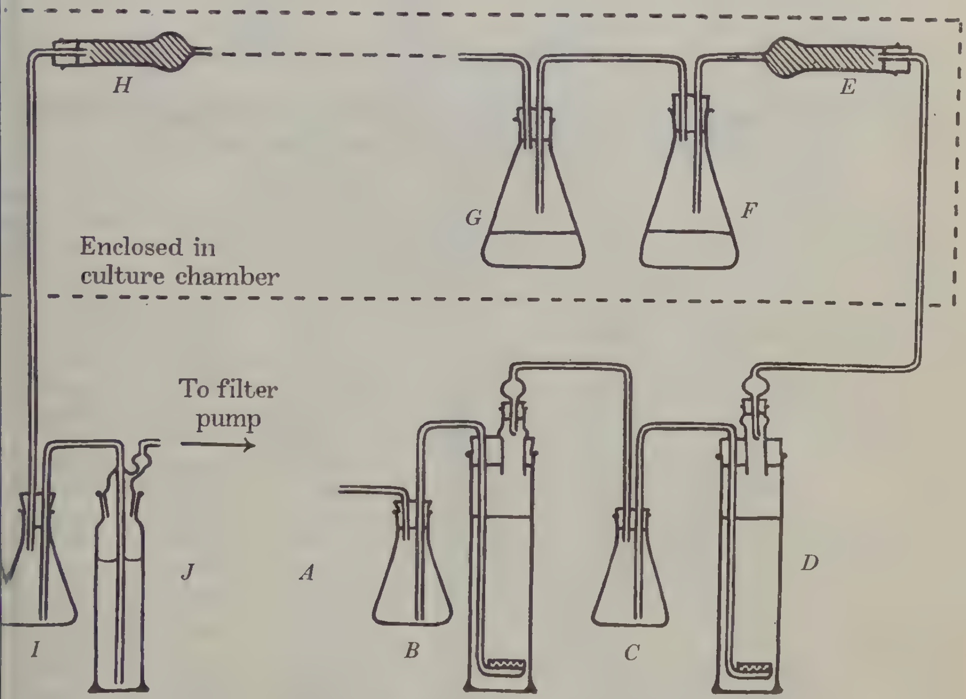

Fig. 1. Diagram of the aeration apparatus.

The cultures were inoculated from a pure bacteria-free stock culture. In order ensure that the cultures had not become contaminated during the course of the periment a small amount of material from each of the flasks was examined cteriologically at the end of the experimental period by the same means as those ed for the examination of stock cultures. No contamination by fungi or bacteria s found.

The results of micro-Kjeldahl analyses of the cultures are given in Table 1.

This table shows clearly that free nitrogen has been fixed by the alga. The small ount of nitrogen found in the control flask, the medium in which was cometely free from combined nitrogen at the beginning of the experiment, may rhaps be due to ammonia derived from the culture flasks. It is to be noted that a large proportion of the nitrogen fixed appears in a soluble form in the medium. The nature of this excreted nitrogen is being investigated.

Table 1. *Nitrogen fixation by* Anabaena cylindrica *in aerated culture after* 50 *days. Alga showing no sign of senescence.* 100 *ml. nitrogen-free medium per flask. Nitrogen in mg.*

	Alga	Medium	Total
Control	—	0·019	0·019
1	1·403	0·093	1·496
2	1·163	0·073	1·236
3	1·878	0·133	2·011

Table 2. *The changes produced by* Anabaena cylindrica *in a medium containing potassium nitrate.* 75 *ml. medium per flask. Nitrogen in mg.*

Age of culture	N in alga	Total N	Deviation of total N from control	Nitrate N in medium	Total N in medium	Other N in medium (by difference)
Control	—	1·243	—	1·243	1·243	—
	—	1·371	—	1·371	1·371	—
Means	—	1·307	—	1·307	1·307	—
20 days	0·173	1·328	+0·014	1·114	1·155	0·041
	0·193	1·359	+0·052	1·167	1·166	0·000
	0·212	1·392	+0·085	1·134	1·180	0·046
Means	0·193	1·360	+0·050	1·138	1·167	0·030
30 days	0·332	1·324	+0·017	0·861	0·992	0·131
	0·326	1·261	−0·046	0·908	0·935	0·027
	0·369	1·373	+0·066	0·932	1·004	0·072
Means	0·342	1·319	+0·012	0·900	0·977	0·077
40 days	0·488	1·126	−0·181	0·533	0·638	0·108
	0·685	1·278	−0·029	0·503	0·593	0·093
	0·450	—	—	0·521	—	—
Means	0·541	1·202	−0·105	0·519	0·615	0·100
50 days	1·384	1·621	+0·314	0·160	0·237	0·177
	1·161	1·593	+0·286	0·189	0·432	0·243
	1·368	1·629	+0·322	0·006	0·261	0·255
Means	1·304	1·614	+0·307	0·085	0·310	0·225

4. INHIBITION OF NITROGEN FIXATION BY COMBINED NITROGEN

As in other nitrogen-fixing organisms, nitrogen fixation by *Anabaena cylindrica* does not take place in the presence of a sufficient amount of readily available combined nitrogen. The changes taking place in cultures supplied with nitrate have been followed up experimentally. Cultures of *Anabaena* were set up in a medium containing nitrate and samples of three, chosen at random, analysed at intervals. The nitrogen in the alga, the total nitrogen in the medium and the amount of nitrate remaining were determined. The results are given in Table 2 and Fig. 2 and may be summarized as follows:

(1) As the nitrogen in the alga increases there is a corresponding decrease in nitrate nitrogen.

(2) Extracellular nitrogen other than nitrate increases as the cultures grow older, nounting to about 14 % of the total nitrogen after 50 days.

(3) Total nitrogen remains roughly constant until the nitrate is nearly exhausted, us showing that nitrogen fixation does not take place in the presence of this form combined nitrogen. Total nitrogen decreases slightly between 20 and 40 days,

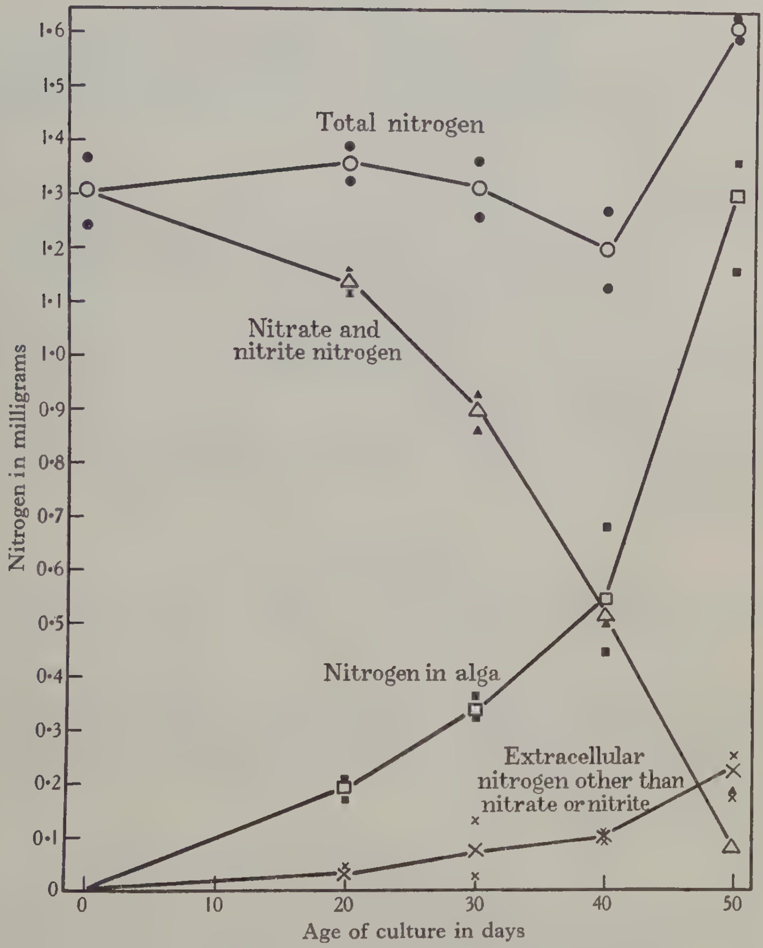

Fig. 2. The changes occurring in cultures of *Anabaena cylindrica* in a medium containing nitrate.

the decrease is not statistically significant. If this decrease is real, it may be to denitrification or conversion of fixed nitrogen to a form which is missed by Kjeldahl method. Nitrogen fixation takes place from 40 to 50 days, by which e the nitrogen in the medium has dropped to about 4 p.p.m.

A similar experiment in which ammonium chloride was used as the source of ogen gave comparable results.

5. CONCLUSION

The work described above shows clearly that *Anabaena cylindrica* is able t fix nitrogen. Four species of *Anabaena* have now been shown to possess thi capacity (De, 1939). It is only in this genus and in species of *Nostoc* (for reference see De, 1939) that reliable evidence is available of the capacity of assimilatin nitrogen in the elemental form. *Nostoc* and *Anabaena* are, however, comparativel easy to work with under laboratory conditions, while other blue-green algae ar more difficult to obtain free from contaminants. Further work may possibly show that the property is more widely distributed in the Myxophyceae than would appea at present.

Anabaena cylindrica was isolated from an aquatic habitat. This alga has not been reported as occurring in any quantity in fresh waters and it probably plays no very important part as a fixer of nitrogen. Other species of *Anabaena*, however, occur in great abundance in certain types of fresh water and may be of considerable importance in this respect. Hutchinson (1941) has reported nitrogen fixation by a planktonic *Anabaena* which, however, was not free from bacteria. Further critical work on this and similar species is necessary.

Even if an alga has been shown to fix nitrogen it is quite possible that the concentration of available combined nitrogen in a given habitat will be sufficient to inhibit the process.

6. SUMMARY

1. *Anabaena cylindrica* Lemm. has been obtained in pure unialgal bacteria-free culture.
2. Due precautions having been taken against contamination by other organisms and error due to absorption of fixed nitrogen from the atmosphere, this alga has been shown to possess the capacity of fixing nitrogen.
3. Nitrogen fixation does not take place in the presence of a sufficient quantity of readily available combined nitrogen.

This work was commenced in the Department of Botany, Queen Mary College, London, and has been partly carried out while the author was receiving a maintenance allowance from the Department of Scientific and Industrial Research. I would like to express my gratitude to Prof. F. E. Fritsch, under whose supervision the investigation has been carried out, and to Mr G. E. Briggs and Dr F. M. Haines for valuable suggestions and criticism. My thanks are also due to Dr W. J. Dowson for advice concerning the bacteriological examination of cultures, to Drs H. G. Thornton and H. Nicol with whom I have discussed methods of obtaining pure cultures of blue-green algae, and to Dr H. D. K. Drew for loan of the micro-Kjeldahl apparatus.

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OBSERVATIONS ON THE DISTRIBUTION OF ASCORBIC ACID (VITAMIN C) IN EXPLANTED CELLS

BY S. A. BARNETT[1]

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(With One Text-figure)

A SURVEY was recently made of the distribution of ascorbic acid in the embryonic tissues of the chick (Barnett & Bourne, 1941, 1942). Its results raised a number of problems which can only be solved experimentally. A study was therefore undertaken of ascorbic acid in cultured tissues from chick embryos. Owing to the war this work could not be completed, but it is considered desirable to record the few observations which have been made.

1. METHODS AND MATERIAL

Cultures were set up in 'hanging drops' according to the method described by Willmer & Kendal (1932). The medium was composed of equal parts of fowl plasma and Compton-Pannet saline containing 15 % embryo juice. All the cultures examined for ascorbic acid were in their third, or a later, passage: that is, they had been recultured at least twice. Reculturing was carried out at 2-day intervals. The observations were made between 24 and 48 hr. after reculturing, on cells at or near the periphery of the outgrowth. No difference was observed between the 24 and 48 hr. cultures in respect of the ascorbic acid content of the cells.

Ascorbic acid was detected by means of the acid silver nitrate method (see Bourne, 1936). The method is based on the fact that, of all intracellular reducing agents, ascorbic acid is the only one which reduces silver nitrate in acid solution, in the dark, over short periods of time. The evidence for the validity of the method is fully discussed by Barnett & Bourne (1941). The reagent used for combined fixation and impregnation was a 10 % solution of silver nitrate in 10 % acetic acid. The cover-slip bearing a culture to be examined was removed from its cavity slide, dipped in distilled water, and then left in acid silver nitrate for about 10 min. After fixation it was washed thoroughly in distilled water, dehydrated and mounted in a temporary medium. In some instances the preparations were toned with gold chloride before dehydration.

The tissues used were 10-day ventricle (15 cultures), 11-day frontal bone (25 cultures), and 17-day adrenal cortex and medulla (40 and 20 cultures respectively). *L*-ascorbic acid (Roche) was added to the medium used for some of the adrenal cultures: it was weighed in crystalline form and added to the saline in concentrations of 2–8 mg. per 100 ml.

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2. RESULTS

In heart fibroblasts no ascorbic acid was detected.

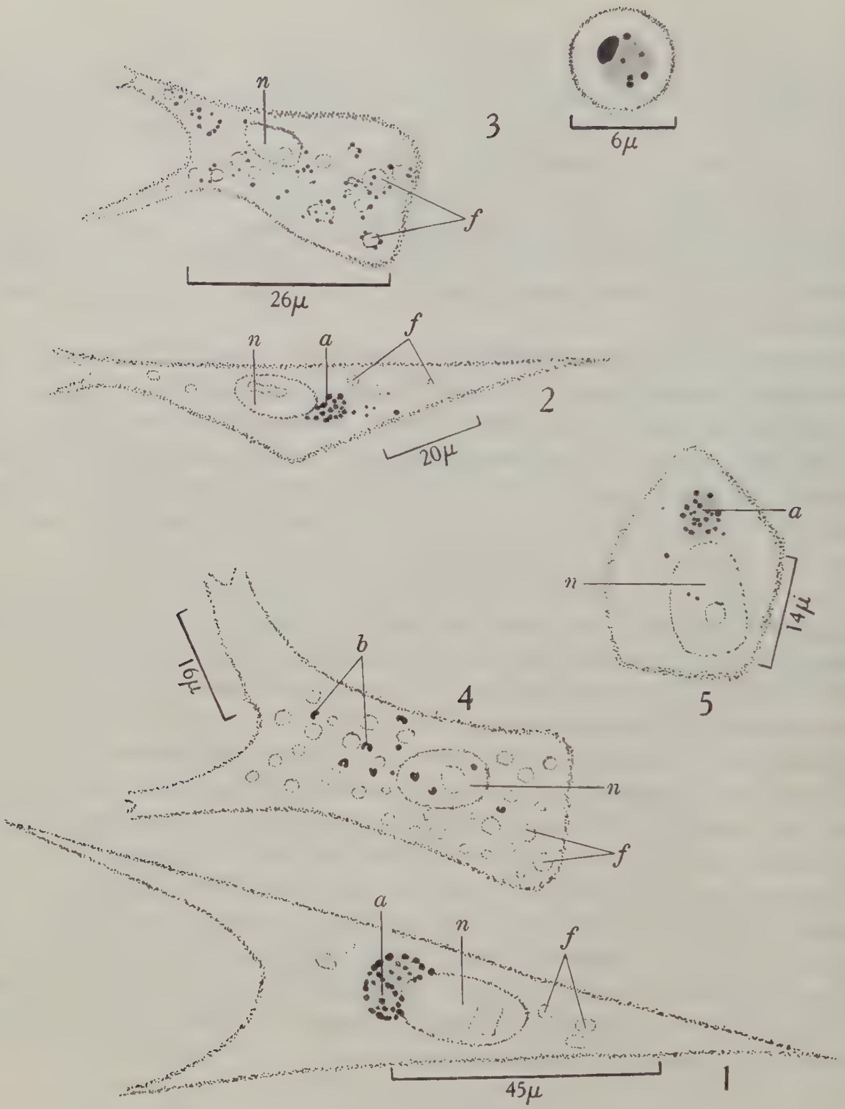
In the bone cultures most of the cells of the outgrowth gave no reaction; this as the case in all those undergoing division. A very small number of cells showed black precipitate in a localized area of cytoplasm adjoining the nucleus (Fig. 1). here is little doubt that this is the region of the Golgi material (Ludford, 1927, 935). The appearance in these unstained preparations is of a greyish mass quite stinct from the clear nuclear area, and from the cytoplasm, on or in which are e black granules of precipitated silver. The cells in which this was observed were herwise normal.

In cultures of adrenal cortex again a majority of the outgrowing cells contained detectable ascorbic acid. However, some normal 'fibroblasts' were observed th local deposits similar to those of the bone cells which contain ascorbic acid ig. 2); again, none of these was dividing. The proportion of these cells was creased, though not very greatly, by the addition of ascorbic acid to the medium. these cultures there occur a few cells of atypical shape containing much fat in e form of globules: they are evidently degenerate, that is, in the process of dying udford, 1935). They usually contain rather large quantities of ascorbic acid, dicated by a granular precipitate associated with the fat (Fig. 3). The globules emselves, with the silver deposits on them, resemble quite closely the yolk anules described by Barnett & Bourne (1941) as occurring in and below the astoderm of early chick embryos.

Cultures of adrenal medulla produce mixed outgrowths containing both fibro- st-like cells and epithelium. The former were only observed to contain ascorbic d when in a degenerate state: the cell drawn in Fig. 4 is an example; the silver posits are unusual in being not in the form of spherical granules, nor localized, t U-shaped and scattered through the cytoplasm. The impregnation has the pearance of a Golgi network which has broken up; such fragmentation of the lgi material has been observed in cultured cells by Ludford (1927) and Richard- n (1934). In addition to the degenerate cells a few epithelial cells show the sence of ascorbic acid: the silver deposit is in a restricted area of the cytoplasm nilar to that observed in the fibroblast types.

3. DISCUSSION

In plant seeds the appearance of large quantities of ascorbic acid coincides with initiation of rapid growth in the tissues concerned (Bonner & Bonner, 1938). ere is no reason to think that anything comparable occurs in animals. The ues used in this study were cultured in a medium optimum for the growth of ck periosteoblasts (Willmer & Jacoby, 1936); very few cells of the outgrowth tained detectable amounts of ascorbic acid, and of these some were isolated and ng: none was dividing. Similarly, in whole chick embryos during the first ays of incubation no ascorbic acid can be found except in the extra-embryonic ues (Barnett & Bourne, 1941). It is evident that histologically demonstrable



The figures are drawings of the cells as seen by means of a 1/12 in. oil immersion objective.

Fig. 1. Normal fibroblast-type cell from 11-day frontal bone, showing a localized impregnation.

Fig. 2. Cell similar to that of Fig. 1, from 17-day adrenal cortex.

Fig. 3. Degenerate cell from adrenal cortex, with granular precipitate associated with fat globules. Only the fat visible in optical section is drawn. The smaller drawing represents a single fat globule, bearing a precipitate, from the same cell.

Fig. 4. Degenerate cell from adrenal medulla, with precipitate evidently associated with fragmented Golgi material.

Fig. 5. Epithelial cell from adrenal medulla, with localized impregnation.

Lettering: a = area of precipitation, presumably Golgi material; b = fragmented material bearing precipitate; f = fat granules; n = nucleus.

scorbic acid need not be present for the occurrence of any of the synthetic processes
nvolved in the multiplication of embryonic chick tissues.

A further question arises in connexion with the site of the silver deposits in the
ells. In those illustrated in Figs. 1, 2 and 5 the deposits are evidently associated
ith the Golgi substance. This association has been frequently observed in the
ast, and has been assumed to show that ascorbic acid itself is present in the Golgi
aterial (see Bourne, 1935; Giroud, 1938). This is not necessarily the case. Pre-
ipitates of this kind often accumulate at the interface between two immiscible
quids, or between a solid and a liquid: the familiar silver mirror is an example.
t is possible that a 'mechanical' effect of this kind is responsible for some at least
f the localized deposits observed. Bensley has made a similar suggestion in con-
exion with osmium impregnations of Golgi substance (Owens & Bensley, 1929).
f this effect does occur in acid silver nitrate preparations the presence of a pre-
pitate indicates only that ascorbic acid was somewhere in the cell; the exact site
the precipitate must then depend on the existence of a certain type of interface.

4. SUMMARY

Heart, bone and adrenal cells from chick embryos were cultured in growth-
romoting media in vitro; their ascorbic acid (vitamin C) content was investigated
y the acid silver nitrate method. Few cells were found to contain ascorbic acid;
ose containing most were degenerate; others showed a silver precipitate in the
olgi substance. The results are discussed.

The author is indebted to Prof. E. S. Goodrich and Prof. W. E. Le Gros Clark
r facilities provided in the Departments of Zoology and Human Anatomy,
xford; and to the Christopher Welch Trust for grants for the purchase of apparatus.

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DIURNAL FLUCTUATIONS OF OXYGEN AND *p*H IN TWO SMALL PONDS AND A STREAM

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(With Two Text-figures)

THERE are few available data on diurnal changes in chemical and physical factors in fresh-water environments. Morren & Morren (1841) are credited with the first observation of a diurnal fluctuation in dissolved oxygen.

A certain amount of work has been done on *lakes*. Birge & Juday (1911) found that at $1\frac{1}{2}$ m. depth the highest values for the oxygen content of the water were obtained in the evening. These observations were made on Lake Mendota (Wisconsin, U.S.A.) during September. Scott (1924) records diurnal changes up to 3 c.c./l. of dissolved oxygen for water among the plants of the littoral regions of Eagle (Winona) Lake, U.S.A. These observations were made during July and August, and the maximum oxygen concentration was observed at 17.00 to 18.00 hr., but the actual data are very incomplete. Philip (1927) records changes of *p*H in a lake in Minnesota (U.S.A.) for the period September to December; the greatest variation was *p*H 7·40 at 07.00 hr. to *p*H 8·95 at 16.00 hr. Czezegy (1938) found an oxygen pulse in Lake Balaton (Hungary), obtaining the maximum of 130 % saturation with oxygen at 16.35 hr. and the minimum of 80 % saturation at 24.00 hr., but readings were only taken at 4 hr. intervals.

There are very few records for the changes of oxygen concentration and *p*H in *ponds*. Cowles & Brambel (1936) give values for the *p*H and the dissolved oxygen of a bog pond. Data were only obtained at 4 hr. intervals during the 24 hr. period and the water samples were taken at different depths. The data appear insufficient and the fluctuations are quite irregular. For marine tidal pools in South Africa, Stephenson, Zoond & Eyre (1934) found changes up to 9 c.c./l. taking place over a period of 4 hr. when extensive growths of algae were present in the pools. Measurements were only made during the intertidal periods, but both a rise of oxygen concentration during daylight and a fall during the hours of darkness were noted.

Apart from observations on enclosed bodies of water there are some data in the literature for the fluctuations of oxygen concentration and *p*H of *running waters*. Butcher, Pentelow & Woodley (1927) showed a definite diurnal change of both ygen concentration and *p*H in two rivers in Sussex. The more organically polluted er showed a daily variation of over 3 c.c./l., while even the rapidly flowing and polluted river exhibited a change of 3 c.c./l. In both rivers the oxygen content s at a minimum in the early morning and at a maximum in the afternoon. In a er in France, Hubault (1927) found an influence of the temperature of the river ter on its oxygen content. Near the source, where the volume of water in the er was small and the daily variation in water temperature large (7·8° C. at .30 hr. to 13·2° C. at 13.15 hr.), the oxygen content was higher in the morning n in the afternoon, the values being 7·52 and 6·37 c.c./l. respectively. No preciable phytoplankton was present in this part of the river, but in the lower ches, where the volume of water was greater, the temperature variation was aller and algae and bacteria were present. Here the oxygen content was at a ximum of 5·35 c.c./l. in the afternoon (at 15.35 hr.) and at a minimum of 6 c.c./l. in the early morning (at 03.45 hr.). In the more recent work of Butcher, ngwell & Pentelow (1937) on the River Tees it is shown that the diurnal variation oxygen and *p*H in the upper reaches depends on the presence of the plant *dophora* in large quantities during spring and early summer. No appreciable rnal variation was found in March and August, when the plant was absent, ereas in July, when the plant was present, the oxygen concentration varied from % saturation at 15.00 hr. to 60 % saturation at 05.00 hr. and the *p*H varied from 5 at 16.00 hr. to 7·65 at 07.00 hr. In addition these authors found a diurnal iation of inorganic nitrogen in the absence of the plant, which they were able correlate with the presence of greater numbers of saprophytic bacteria in the er during the hours of darkness. They were unable however to correlate the rnal fluctuations of bacteria with those of oxygen.

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[1] The work described in this paper was done as a result of some preliminary observations by Dr R. F. Ewer and was carried out jointly with her and Dr C. A. Wingfield.

The object of the present work was to investigate physico-chemical conditions number of fresh-water habitats. The results show the extent to which fluctuas are variable and how isolated samples of water may give quite an erroneous a of the conditions prevailing in the habitat at other times of the day or in other nths of the year. The diurnal oxygen pulse probably depends on the amount of etation present, on the intensity of the sunlight and its penetration into the er, on the water temperature and on the extent of the fauna and other oxygensuming factors.

It was intended to make a series of 24 hr. observations on a number of different ds of ponds at different times during the year and the results recorded in this er were obtained as part of this scheme. Now, however, it appears unlikely that work can be extended in the near future, but it is thought desirable to publish results in spite of their incomplete nature in the hope that they may be of vice to others and that they may draw attention to the desirability of collecting her data.

The methods employed in taking samples from ponds were designed to ensure minimum disturbance of the water. This is an important point when frequent ples of water are taken from small bodies of water in which the oxygen contration and other physico-chemical factors may be in a stratified condition.

The sampling apparatus used on the ponds is illustrated in Fig. 1. It consi of a wooden cradle which could be suspended by the rope *A* immediately over position in the pond from which the samples were to be taken. The other end the rope *A* passed over a pulley, which was made fast to an overhanging bran or to a rope stretched tightly across the pond, to the bank. By the manipulation *A* the cradle could be gently raised from or lowered into the water at the sampli site. The rope *C* served to draw the cradle to the side of the pond when the cra was raised out of the water. The disk marker *B* indicated the depth to which t cradle was sunk in the water and the lead weight *D* was provided to overcome t buoyancy of the apparatus.

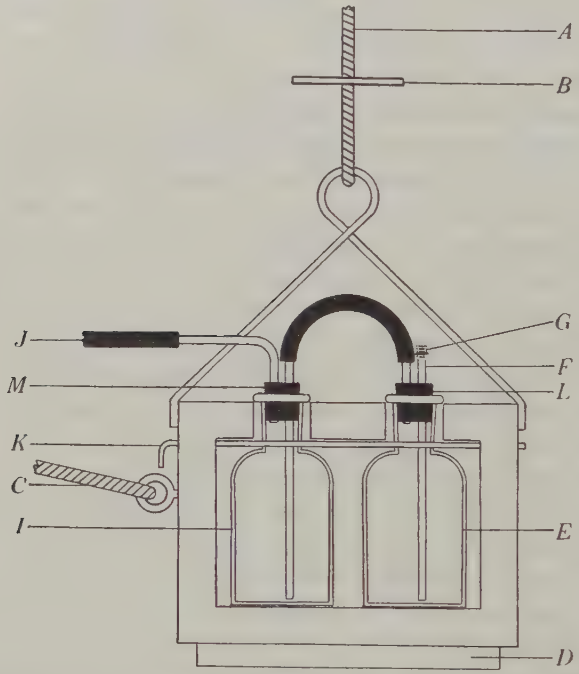

Fig. 1. Cradle apparatus for the collection of water samples. Explanation in text. The fittings t the bottles are represented as being seen by transparency. Rubber fittings are indicated in black.

Two water samples were required for each hour during the 24 hr. period. Abou half an hour before the sampling time the cradle was drawn to the side of the pon and the two bottles, *I* and *E* (Fig. 1), were placed in the cradle and locked i position by the rod *K*. The ground-glass stoppers of the bottles were removed an replaced by the rubber bungs *L* and *M*. (The connexions between the bottles wi be clear from Fig. 1. The pressure tubing *J* is long and connects the bottle *I* wit a 2 l. bottle, at the pond side. The tubing communicates with the 2 l. bottle by stopcock.) The cradle was then swung into position and gently submerged to th required depth as indicated by the disk *B*, the distance between *B* and the openin of the tube *F* into the bottle *E* being the depth of sampling. At the time of samplin the 2 l. bottle on the pond side was evacuated by a pump (a converted bicycle pum vas used) with the stopcock closed. On opening the stopcock the bottles *I* and *E* vere evacuated and water drawn in at *F*. Entry of large plant particles, etc., was revented by the plankton netting cap, *G*, on the tube *F*. The arrangement of tubes n the bottles ensured the complete filling of the bottles without air bubbles. The ample bottles were of 150 c.c. capacity, and when about 1 l. of water had been rawn over into the 2 l. bottle it was fairly certain that the water then filling the ample bottle had not been in contact with the air which previously filled them. he stopcock on the 2 l. bottle was then closed, the cradle was brought to the side f the pond, the sample bottles were removed from the apparatus and immediately losed with their ground-glass stoppers. The cradle was then reassembled with resh sample bottles ready for the next sampling of the pond.

The water temperature at the time of sampling was determined by a thermo-eter enclosed in a wide-bore glass tube to provide an air-jacket. The thermo-eter thus lagged was clipped on to the cradle so that its bulb was on a level with *G*.

One of the water samples was used for the determination of the oxygen content. his was done by means of the Winkler technique employing the Rideal-Stewart odification to avoid errors due to organic substances which may be in the water *Standard Methods of Water Analysis*, 1936). The analysis was done in the field ithin half an hour of collection of the sample.

The other water sample was used for the determinations of *p*H and alkalinity. hese were also done within half an hour of sampling. The *p*H was determined by eans of the B.D.H. Lovibond Comparator, and the alkalinity by titration of the ample against standard sulphuric acid solution employing methyl orange as an dicator. The alkalinity result is expressed as a normality.

Two small ponds and a small, rapidly flowing stream were investigated. One f the ponds, Selly Park Pond, Birmingham,[1] was taken as an example of ponds of ow alkalinity. The other pond, Grange Farm Pond, Alvechurch, Worcestershire, as of relatively high alkalinity. The stream investigated is a small tributary of the iver Stour at Blakedown, Worcestershire.

Observations at Grange Farm Pond, Alvechurch, Worcestershire, were made on –4 June 1937. Samples were taken 12 in. below the surface from a section of the ond 1½–2 ft. deep, covered with duckweed and in the shadow of trees. The weather as dull, overcast and showery. The alkalinity varied between 0·0041 and 0·0043 N, e *p*H between 7·5 and 7·9 and the temperature between 12·6 and 13·7° C.

Further data were obtained from the same pond on 23–24 June 1937. Samples ere taken 6 in. below the surface from a section of the pond 1½ ft. deep. The water as not permanently covered with duckweed and was unshaded by trees. The eather was sunny and cloudless at first, but from 10.00 hr. until about 16.00 hr. ere were heavy showers of rain interspersed with bright and overcast periods. The kalinity variation was 0·0044–0·0048 N, the *p*H 7·2–7·5 and the temperature 2·7–14·7° C.

Observations at Selly Park Pond, Selly Park, Birmingham, were made on 9–30 September 1937. Samples were taken 6 in. below the surface. The pond was fairly well shaded and the surface entirely covered with vegetation: *Potamogeto natans*, duckweed and filamentous algae. Weather was dull with bright interva and a little sunshine. The alkalinity was 0·0026–0·0027 *N*, the *p*H 7·0–7·2, and th temperature 11·4–12·1° C. Further records were made at the same pond o 11–12 April 1938. Samples taken 6 in. below the surface. The pond was fairly we shaded by trees and the surface thickly covered with vegetation, of which filamentous algae were the most abundant. Weather generally warm and sunny durin the day. The alkalinity was 0·0019–0·0021 *N*, the *p*H 7·7–8·7 and the temperatur 5·8–6·8° C.

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[1] This is the upper pond in the garden of a house known as 'Highfield' in Selly Park Road.

Observations on a tributary of the River Stour at Blakedown, Worcestershire were made on 5–6 February 1938. Samples were taken within 2 in. of the stream bed and in midstream. The stream was in the shade and, in the section investigated the water current was rapid and there was no aquatic vegetation. The weather wa generally dull and overcast. The alkalinity was 0·0022–0·0028 *N*, the *p*H 7·5–8·0 and the temperature 6·6–7·0° C.

The diurnal variations in dissolved oxygen in the various environments are shown in Fig. 2. Oxygen concentration is expressed in c.c./l. and not, as is often the case, as percentage saturation. This is desirable because in such a system as a pond we are not dealing simply with oxygen absorption and loss at the water-air surface but also with its production and utilization by the organisms in the water. It would appear that the latter factors are the more important in producing the oxygen pulse, at any rate in the presence of a well-developed flora and fauna, for the oxygen concentration is usually found to fall during the night when the temperature is falling and the capacity of the water for taking up oxygen from the atmosphere is increasing. The low oxygen concentrations found for Selly Park Pond in September also seem to indicate that absorption of oxygen from the air is a minor factor, since the values were much below the air saturation value for the particular temperature. It was found that a uniform temperature was often maintained for several hours, during which time the oxygen concentration altered considerably. Since, therefore, gaseous interchange is obviously not the determining factor in the oxygen balance in the pond it would seem best not to use a measure such as percentage saturation.

It will be seen from Fig. 2 that the maximum changes in oxygen concentration recorded are of the order of 2–3 c.c./l. and occurred in the spring observations on the Selly Park Pond and in the summer observations of the Grange Farm Pond. The maxima occur just about or slightly after dark, and the minima shortly after dawn. There would thus seem to be a time lag in the appearance of the oxygen resulting from the period of greatest photosynthetic activity, which was presumably about midday when the intensity of the sunlight was greatest. During the hours of darkness there was a gradual reduction of the oxygen content due to the respiration of all the organisms in the pond. In the Selly Park Pond in September the oxygen content throughout the 24 hr. period remained consistently low, although there was a slight diurnal rhythm involving an increase of about 0·4 c.c./l., the maximum occurring as usual soon after dark (Fig. 2). The difference in the oxygen pulse of the elly Park Pond for April and September is therefore very considerable and the asons for it are by no means clear. The temperature was higher and the day, if ything, more sunny in September than in April, while on both occasions much uatic vegetation was present. It may be that in September the vegetation was

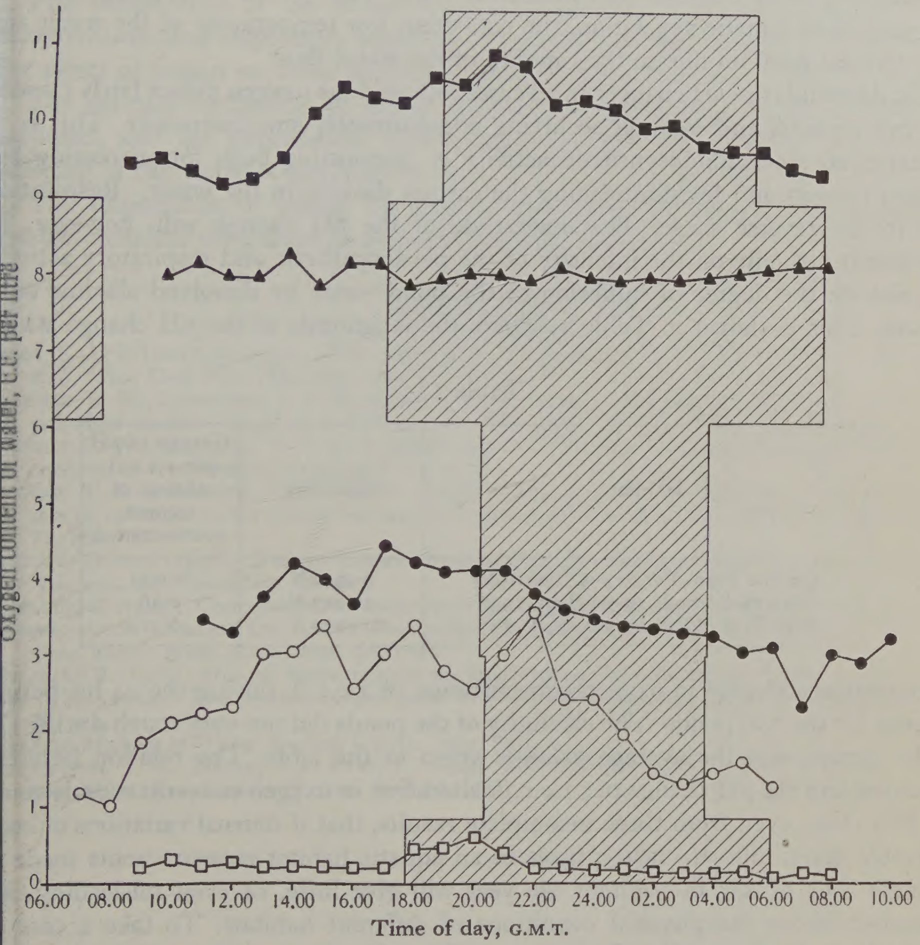

Fig. 2. Fluctuations of oxygen concentration during 24 consecutive hours for all the habitats studied. Hours of darkness for each curve represented by shading.

■ Selly Park Pond, 11–12 April 1938.
▲ Blakedown Stream, 5–6 February 1938.
● Grange Farm Pond, 3–4 June 1937.
○ Grange Farm Pond, 23–24 June 1937.
□ Selly Park Pond, 29–30 September 1937.

eady beginning to die down and its photosynthetic activity therefore diminished, t of this we have no proof and further investigation is required. It is also possible t the decomposition of organic matter in the mud at the bottom of the pond may a factor of importance in this difference. Another curious fact is that the oxygen tent in the Grange Farm Pond on 3 June was consistently higher than it was

20 days later on 23 June, although in the former case the samples were collecte in the shade and in the latter case in the sunlight.

The data for the Blakedown Stream during February present no features o particular interest. There is no evidence of a diurnal variation, and the oxyge content was fairly constant and uniformly high. This would be accounted for b the scarcity of aquatic vegetation, the consistent low temperature of the water an the vigorous aeration due to the rapidity of the water flow.

In the ponds the *p*H changes in all cases followed the oxygen pulses fairly closely the two maxima and minima occurring approximately simultaneously. This is t be expected since photosynthetic activity is responsible both for increasing th oxygen present and for diminishing the carbon dioxide in the water. Respiratio acts in the reverse sense. The magnitude of the *p*H change will, however, b dependent not only on the intensity of the photosynthetic and respiratory activit but also on the degree of buffering of the pond water by dissolved alkaline substances. This is shown in Table 1, where the magnitude of the *p*H change whic accompanies a change in oxygen concentration of 1 c.c./l. during the 24 hr. period is given for the two ponds. The alkalinity of the ponds did not vary much during the 24 hr. period and the average value is given in the table. The relation between alkalinity and the *p*H change per 1 c.c./l. alteration in oxygen concentration is clear.

Table 1

Habitat	Alkalinity	Change of *p*H per 1 c.c./l. change of oxygen concentration
Grange Farm Pond, 3–4 June 1937	0·0042 *N*	0·05
Selly Park Pond, 29–30 Sept. 1937	0·0027 *N*	0·36
Selly Park Pond, 11–12 April 1937	0·0020 *N*	0·51

It is clear, even from these incomplete results, that if diurnal variations of considerable magnitude are taking place in an aquatic habitat measurements made at a single time of day throughout the year will give little, or even misleading, information about the physical conditions of different habitats. To take a case in point, suppose the conditions of Grange Farm Pond on 23 June were compared with those of Selly Park Pond on 29 September by means of single samples taken in both cases at 09.00 hr. Both would be classified as low-oxygen habitats, the difference between the oxygen concentrations being less than 1 c.c./l. Yet if the habitats were compared by means of single samples taken in both cases at 15.00 hr. a difference of oxygen concentration of over 3 c.c./l. would be revealed. Evidence has also been given above that the oxygen pulse of a single pond may show considerable seasonal variation.

SUMMARY

Data are given of the diurnal fluctuations of oxygen, *p*H and temperature in o ponds and a stream. One of the ponds was studied at two different seasons.

The dependence of the oxygen concentration and its diurnal fluctuation on otosynthetic and respiratory activities of organisms of the habitats is pointed out. he effect of season on these activities was very marked in the one case studied.

The diurnal fluctuation of *p*H in relation to that of oxygen concentration is scussed, and the dependence of the degree of *p*H fluctuation on the natural ffering of the water is pointed out.

The importance of taking the diurnal pulse into account in comparing the ysico-chemical conditions of different habitats is stressed.

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